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CHARACTERIZATION OF HUMAN TUMOUR CELL LINES WITH DIFFERENT RADIOSENSITIVITIES

by

Sami S. Qutob, M.Sc.

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in
Pharmacology

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All studies reported in this thesis were conducted under the supervision of Dr. Cheng E. Ng. Unless otherwise indicated all experimental work was carried out by Mr. Sami S. Qutob. Mr. Sami S. Qutob and Dr. Cheng E. Ng wrote all manuscripts in this thesis unless otherwise indicated.

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assisted in UV and tumour growth studies. Master's student, Ms Dana Mullins also provided assistance with the growth of the clones in mice. Mr. Sami S. Qutob completed the majority of the experimental work and data analysis under the supervision of Dr. Cheng E. Ng.

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Authors: Sami S. Qutob, Felix M. Mesak, Denise Proulx, Qing Y. Liu, and Cheng E. Ng

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GENERAL ABSTRACT

Failure of radiotherapy for treatment of neoplastic disease can be due to selection of radioresistant S-phase cells within tumours. However, S-phase cells can be targeted with a type-1 DNA topoisomerase inhibitor, camptothecin (CPT) or one of its analogs. The first objective of this study was to understand the mechanism of cellular death of human tumour and normal cells following treatment to CPT and single dose (non-fractionated) X-radiation. We found that necrotic cell death was more important than apoptotic cell death during concurrent CPT and radiation treatment in melanoma (Sk-Mel-3) cells, but not in normal fibroblast (AG1522) cells.

Further experiments involving fractionated X-radiation pre-treatment gave rise to the presence of radioresistant cells in the human tumour colorectal cell line (HCT116). Therefore, the second objective of this study was to assess whether the radioresistant cells occurred by selection or adaptation to the X-radiation treatment. We discovered that the resistant subpopulation of cells surviving a previous fractionated irradiation exposure was likely due to the selection of a radioresistant subpopulation, whereas the radiation-sensitive clone manifested a potential inducible radiation response. The third objective was to produce a model system for distinguishing the genetic factors that may be involved in the radiosensitive or radioresistant phenotype. To this end, a set of untreated genetically-related human cell clones of varying radioresponses, with non-overlapping drug sensitivities, were generated from the HCT116 cell line, suggesting the presence of clonal heterogeneity and providing an excellent model. Lastly, using the clones derived from objective 3, the fourth objective was to exploit this model system and determine the genetic basis of radiosensitivity. To do this, we used human cDNA microarrays containing 19,200 ESTs and verified these gene expression changes with Q-PCR. Of a

number of genes identified by the array, only spermidine/spermine N1-acetyltransferase (SSAT), an enzyme that catabolizes radioprotective polyamines had a known potential link to radiosensitivity. Our findings establish the potential importance of intrinsic gene expression in radioresponsiveness, and the identification of SSAT as a possible modulator of radiosensitivity, providing a potential tool for understanding and predicting X-radiation response.

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LIST OF ABBREVIATIONS

λ	Lambda
μg	Microgram
μl	Microlitre
aa	Amino Acid
AG1522	Immortalized Human Fibroblasts
AT	Ataxia Telangiectasia
ATCC	American Type Culture Collection
ATM	Ataxia Telangiectasia Mutated
ATR	ATM Rad3-related
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool Algorithm
bp	Base Pair
BSA	Bovine Serum Albumin
°C	Degrees Celsius
CDDP	Cisplatin
CDK	Cyclin Dependent Kinase
cDNA	Complementary Deoxyribonucleic Acid
CPT	Camptothecin
Cy3	Cyanine 3
Cy5	Cyanine 5
DAPI	2-4-Diamidine-2-Phenyllindole
DENSPM	N^1, N^{13}–Diethylnorspermine
DMEM	Dulbecco's modified essential medium
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent Protein Kinase Catalytic Subunit
DSB	Double Strand Break
EDTA	Ethylenediaminetetraacetate
ER	Endoplasmic Reticulum
EST	Expressed Sequence Tag
FBS	Fetal Bovine Serum
g	Gravity
Gy	Gray (1 Gy = 100 rads)
h	Hour(s)
H_2O_2	Hydrogen Peroxide
HCT116	Human Colorectal Tumour Cells
HDR	High Dose Rate
HPLC	High Performance Liquid Chromatography
IGF	Insulin-like Growth factor
IR	Ionizing Radiation
Kb	Kilo Base
Kda	Kilo Dalton
Kg	Kilogram

L	Litre
LQ	Linear Quadratic
M	Molar
mg	Milligram
min	Minutes
ml	Millilitre
mM	Millimolar
MM	Metastatic Melanoma
mRNA	Messenger Ribonucleic Acid
mt	Mutant
MW	Molecular Weight
N_2	Liquid Nitrogen
Na_2HPO_4	Sodium Phosphate
NaCl	Sodium Chloride
NCGR	National Center for Genome Resources
NCS	Newborn Calf Serum
NHEJ	Non-Homologous End Joining Repair
PARP	Poly (ADP-Ribose) Polymerase
PCR	Polymerase Chain Reaction
PE	Plating Efficiency
PUT	Putrescine
QFISH	Quantitative Fluorescence *In situ* Hybridization
QPCR	Quantitative (Real-Time) Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SF	Surviving Fraction
SF2	Surviving Fraction at a 2 Gy radiation dose
Sk-Mel3	Radioresistant Fibroblast-like Human Melanoma
SLD	Sublethal Damage
SLDR	Sublethal Damage Repair
SPD	Spermidine
SPM	Spermine
SSAT	Spermidine/Spermine N^1-Acetyltransferase
SSB	Single Strand Break
T-25	$25cm^2$ flask
T-75	75 cm^2 flask
TPT	Topotecan
UV	Ultraviolet
v/v	Volume/Volume
w/v	Weight/Volume
wt	Wild Type

VP16	Etoposide
XR	X-radiation
XRCC	X-ray Cross Complementation

GLOSSARY OF RADIATION TERMS

Clonogenic Assay: a proliferative assay used to determine the effectiveness of either radiation or chemotherapeutic agents in cell killing.

Cumulative dose: The total dose resulting from repeated exposures of ionizing radiation over a period of time.

Dose: A general term used to refer to the effect on a material which is exposed to radiation. Often refers to the amount of energy absorbed by a material exposed to radiation or to the potential biological effect in tissue exposed to radiation.

Dose rate: The radiation dose delivered per unit time.

Gray (Gy): The new international system (SI) unit of radiation dose expressed in terms of absorbed energy per unit mass of tissue. The gray is the unit of absorbed dose and has replaced the rad. 1 gray = 1 Joule/kilogram and also equals 100 rad.

Photon: A quantum (or packet) of energy emitted in the form of electromagnetic radiation. Gamma rays and x rays are examples of photons.

Potentially lethal damage (PLD): defined as radiation damage which, if unrepaired, is lethal.

Potentially lethal damage repair (PLDR): The enhancement in survival seen following manipulation of postirradiation conditions.

Rad: The original unit developed for expressing absorbed dose, which is the amount of energy from any type of ionizing radiation deposited in any medium. A dose of one rad is equivalent to the absorption of 100 ergs (a small but measurable amount of energy) per gram of absorbing tissue. The rad has been replaced by the Gray in the SI system of units (100 rad = 1 gray).

Radiosensitivity: The relative susceptibility of cells, tissues, organs, organisms, or other substances to the damaging effects of ionizing radiation.

Radioresistance: A decreased ability of cells to respond to exposure of ionizing radiation.

Sievert (Sv): The international system (SI) unit for dose equivalent equal to 1 Joule/kilogram. The sievert has replaced the rem. One sievert is equivalent to 100 rem.

Sub-Lethal damage (SLD): is damage that is not lethal to the cell on its own but can become lethal if it is allowed to interact with similar damage from a further dose of radiation treatment.

Sub-Lethal damage repair (SLDR): is defined as the enhancement in survival when a dose of radiation is separated over a period of time.

Ultraviolet: Electromagnetic radiation with a wavelength ranging from violet within the visible spectrum to low energy x rays.

X-rays: Penetrating electromagnetic radiation having a range of wavelengths (energies) that are similar to those of gamma photons. X-rays are produced by the excitation of the electron field around certain nuclei producing shifts in the electrons between the rings outside the nucleus of an atom. There is no difference in x-rays and gamma photons, but their origin. For example, gamma photons are produced by radioactive decay originating from within the nucleus of an atom.

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Of course the greatest portion of gratitude goes out to my parents, for the moral support and prayers they have given me as I pursued my academic career. They have taught me as well as my dear brothers Majdi, Rashad and Ahmed, and my dearest sister Dinah, the value of responsibility and working hard to make our dreams become a reality. My Dad has given me these pieces of advice that I would like to share.

1. If you think you are beaten, you are.
2. If you think you would dare not, you don't.
3. If you think you'll lose, you've lost.
4. If you would like to win but you think that you can't it's almost a cinch that you won't.
5. Success begins with a fellow's will.
6. Its all in your state of mind.

It is to my family that I dedicate this thesis and my thanks.

"Science is facts. Just as houses are made of stones, so science is made of facts. But a pile of stones is not a house and a collection of facts is not necessarily science."

Jules Henri Poincare (1854-1912)

CHAPTER 1

GENERAL LITERATURE REVIEW

1. BACKGROUND

Statistics Canada has determined for 2001 that cancers were responsible for 29% of all Canadian deaths (Statistics_Canada, 2003). According to the National Cancer Institute of Canada, approximately 145,500 new cases of cancer and 68,300 deaths from cancer are predicted to occur in Canada for 2004 (McLaughlin, 2004). Cancer is a disease in which a cell becomes abnormal, loses its specific specialization and replicates uncontrollably forming a malignant tumour. The initiation of cancer, like most illnesses, is multifactorial and include factors such as age, genetic predisposition, diet, compromised immunity, viral infection, and exposure to harmful environmental factors (i.e. natural and made-made radiation, tobacco smoke). In principle, cancer can appear anywhere in the body, proliferate rapidly at the location where it initially appears, infiltrate surrounding tissue, and spread or metastasize throughout the body. All types of cancer can be treated and mainly those, which are localized, have the potential to be cured, but relatively few cancers are curable when they have metastasized (Cummins *et al.*, 2004; Feun *et al.*, 1982; Masters and Koberle, 2003).

Radiotherapy has been an important mode of treatment of cancer for over 70 years with about one half of cancer patients today being given radiotherapy (Garcia-Barros *et al.*, 2003; Ringborg *et al.*, 2003). The scientific literature provides substantial information about the effects of radiotherapy for the most common types of cancer, showing that it can be used for either the palliation of symptoms, a complete cure, or can

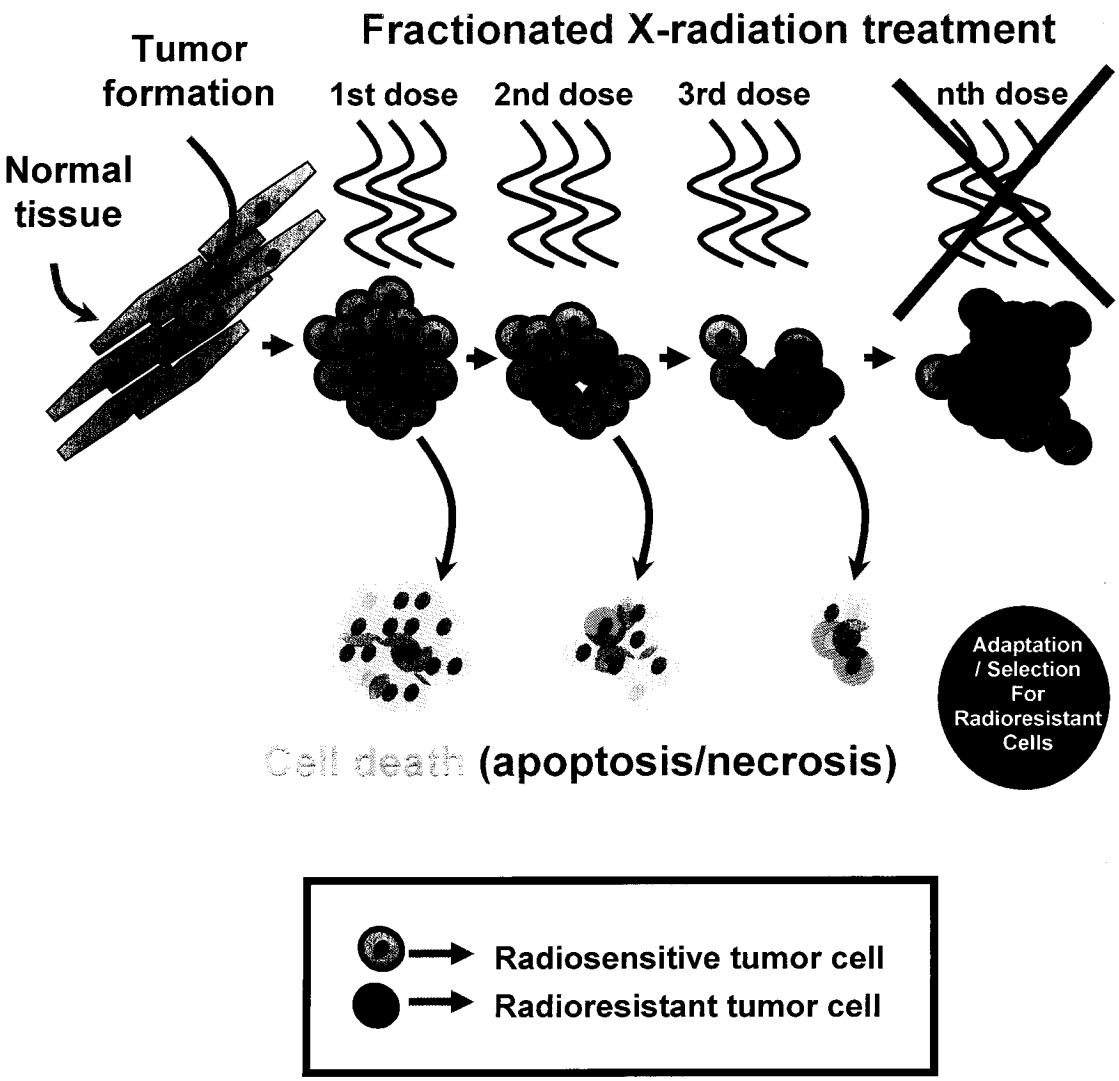
be used, with or without chemotherapy, as adjuvant therapy to surgery. For example, radiotherapy alone can have curative effects on the head and neck region and some lymphomas at early, localized stages (Mitsuhashi *et al.*, 1999). However, with other types of tumours, such as brain malignancies, radiotherapy often provides symptom relief but seldom a cure (Niranjan *et al.*, 2000). In a majority of cases where the tumour is accessible, radiotherapy is often used in conjunction with surgery, often in the neoadjuvant setting, in order to shrink the tumour to a manageable size prior to excision. Tumour types often are treated in the adjuvant setting include many types of cancer including breast cancer, rectal cancer, sarcoma (Munzenrider, 1977), and cervical and uterine cancer (Waggoner, 2003). Furthermore, radiotherapy is shown to have good palliative effects for skeletal metastases and symptomatic metastases in the brain (Schratter-Sehn and Kielhauser, 1991; Sneed *et al.*, 1996). It has also been shown that radiotherapy in combination with chemotherapy may also enhance tumour control (Inciura and Juozaityte, 2002) and as such has found widespread use for treating many cancer types (Curran, 2002).

Regrettably, local regrowth by a subpopulation of cancer cells following radiotherapy is still a major impediment to recovery in many clinical cases. For example, in the Unites States in 2003, the number of patients diagnosed with colorectal cancer was 147,500 with 50% was present with or was developing disease recurrence (Jemal *et al.*, 2003). In another report, seventy-eight patients possessing carcinoma of the uterine cervix, a disease that accounts for approximately one-third of cancers in females

worldwide, were treated with fractionated radiotherapy of five (2 Gy) fractions per week with a total dose ranging from 35 Gy to 50 Gy. Post treatment histological examination of the biopsies revealed that 51% of the patients showed a complete curative response to the radiotherapy indicated by the absence of viable tumour cells, whereas 49% of cases had residual tumours. Further analysis of 38 cases of radioresistant tumours demonstrated that 13 of these tumours had biochemical markers associated with radioresistance and chemoresistance. Of these biomarkers it was established that the pro-apoptosis protein Bax and the proliferative marker minichromosome maintenance (MCM) 2 showed the most significant correlation with predicting radiosensitivity of tumours (Mukherjee *et al.*, 2001).

A multitude of clinical studies have provided evidence that repopulation of a primary tumour by an actively proliferating radioresistant subpopulation of cells can occur during a typical course of fractionated radiotherapy (e.g. 2 Gy x 30 fractions over six weeks) (See figure 1) and were found to be difficult to treat with continual radiotherapy (Hoffman *et al.*, 1967; Tyrsina *et al.*, 1997; Weichselbaum, 1986). One study demonstrating these findings found increased radiation resistance of tumours post radiation therapy than in tumours taken from nonirradiated patients, as assessed by proliferative capacity measured by the clonogenic assay (Weichselbaum *et al.*, 1988).

Figure 1. A diagram illustrating the development of a ionizing radiation-resistant subpopulation of cancer cells following radiotherapy. An overgrowth of radioresistant cells in a tumour occurs because a greater portion of radiosensitive cells die with each radiation dose, compared with radioresistant cells.


Fractionated X-radiation treatment
Tumor formation
1st dose
2nd dose
3rd dose
nth dose
Normal tissue
Adaptation / Selection For Radioresistant Cells
Cell death (apoptosis/necrosis)
Radiosensitive tumor cell
Radioresistant tumor cell

The mechanisms associated with the presence of X-radiation resistant cancer cells following treatment are still under debate. It is still unclear whether radioresistant cells are intrinsically radioresistant (i.e. treatment had merely selected for these cells) or had acquired genetic mutations due to the treatment process (i.e. adaptive response to the treatment). The development of radioresistance have been attributed to include modifications in the normal cell cycle, altered cell growth, impaired DNA repair, and diminished programmed cell death (also known as apoptosis) (Coleman and Stevenson, 1996; Coucke and Crompton, 1995; Maity *et al.*, 1997; Powell and Abraham, 1993b).

1.1 DEFINITION AND QUANTIFICATION OF RADIOSENSITIVITY

Radioresistance refers to the preservation of organ functionality at the tissue level and the ability to preserve viability or reproductive viability (i.e. clonogenicity) at the cellular level following absorption of normally toxic levels of ionizing radiation. The classic assay for the characterization of radiosensitivity is the clonogenic assay which measures the ability of a cell to propagate into a colony starting from a single cell. This assay describes the clonogenic potential of a cell type as a function of the given radiation dose and is regarded as a measure of the overall cell survival (Hall, 2000a). The surviving fraction (SF), represents the fraction of surviving cells following exposure to a radiation dose and is used as a measurement for radiosensitivity. SF(D) is a measurement of the surviving fraction of irradiated cells with a dose (D) normalized to plating efficiency of

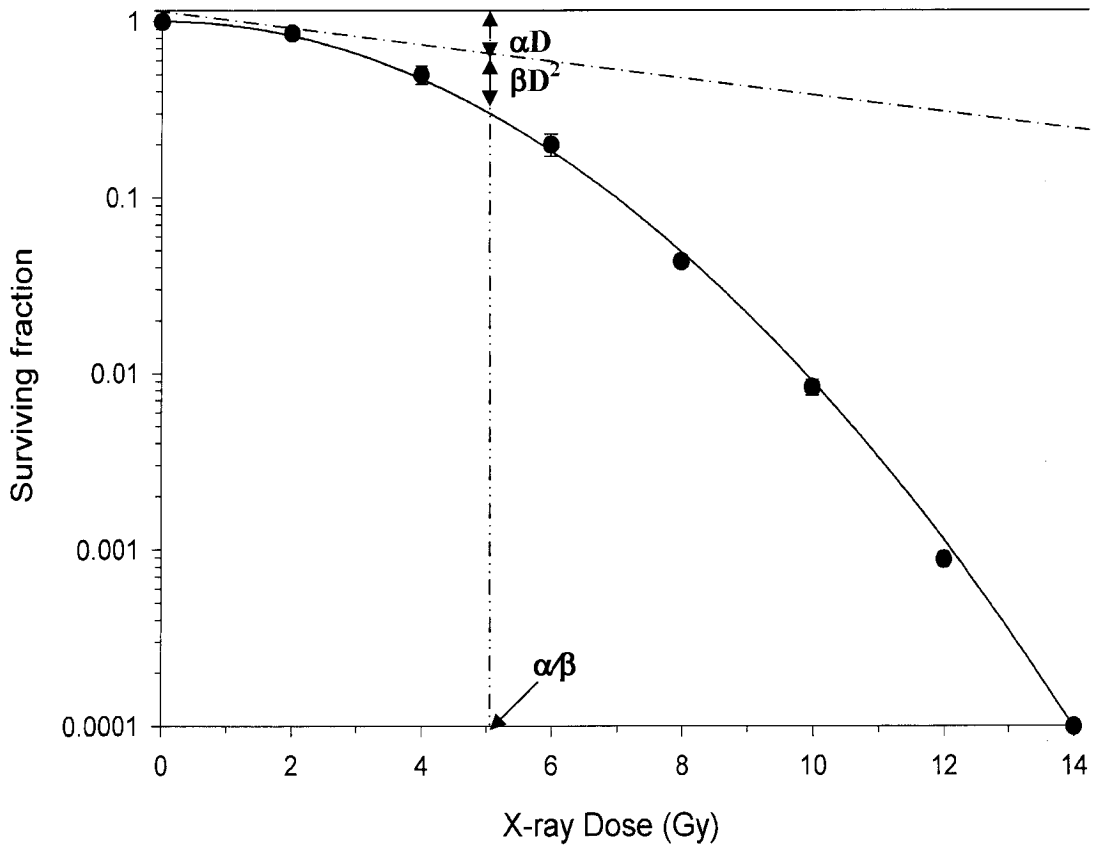
the untreated cells. Therefore, a "SF2 of 0.50" should be read as 50 % of the cells irradiated with 2 Gy maintained clonogenic capacity as compared to untreated cells. A limitation of the clonogenic assay is that it is not possible to perform it for most primary tumour material due to cell senescence under *in vitro* conditions, but it provides a rapid accurate measure of radiosensitivity for cell types that readily form colonies when attached to a substrate.

1.1.1 *Linear-quadratic survival model: a measure of radiation sensitivity or resistance*

The generation of the surviving fraction value is based upon the survival model, also known as the linear-quadratic model, which assumes that there are two components of ionizing radiation-induced *in vitro* cell kill, one proportional to the dose (D) and the other proportional to the square of the dose (D^2) (Hall, 1994). According to this model, the expression for cell survival is based on the equation $\mathbf{S = e^{-(\alpha D+\beta D^2)}}$ where S is the fraction of cells surviving a dose D, and α and β are constants. This model stems from the work of Chadwick and Leenhouts who assumed that the direct production of a DNA double-strand breaks (DSB) leads to an irreversible lethal event (Chadwick and Leenhouts, 1973). The probability of lethality by way of DSB formation caused by a single charged particle track produces the linear component (α) of the equation with the quadratic component (β) of the equation relating to DSBs formed from two distinct ionizing events (Hall, 1994). Therefore, the total probability of lethality (L) is represented

as $\mathbf{L = -\alpha D - \beta D^2}$. A group of cells will have a surviving fraction (SF) of 1 (i.e. 100%) if it contains no lethal events (L = 0) and is presented by the Poisson formula $\mathbf{SF = e^{L} = e^{-(\alpha D + \beta D^2)}}$. According to this formula if $\mathbf{L = 0}$, then $\mathbf{SF = e^{0} = 1}$ (Kellerer and Rossi, 1972; Kellerer and Rossi, 1978). Surviving fractions at various radiation doses are represented by a survival curve generated by the linear quadratic model with the initial slope and the degree of curvature of the fitted line being determined by the constants α and β (See figure 2).

Figure 2. Representation of a typical survival curve associated with radiation therapy. There are two components of cell killing: one is proportional to dose (αD), while the other is proportional to the square of the dose (βD^2). The dose at which the linear and quadratic components are equal is the ratio α/β.


1
0.1
0.01
0.001
0.0001
Surviving fraction
αD
βD²
α/β
0
2
4
6
8
10
12
14
X-ray Dose (Gy)

Normal and tumour tissues are generally classified by their radiobiological characteristics as either late-responding or acutely-responding. These differences in radiobiological behavior are reflected in the ratio of α to β (α/β) values that characterize radiation survival curves. Late-responding tissues have low α/β values while acutely responding tissues have higher α/β ratios. What this tells us is that the lower the radiation dose, given either alone or as per fraction, the increased sparing of tissues with a lower α/β ratio over tissues with higher α/β ratio (Dale and Jones, 1998). Fractionation in clinical radiotherapy relies on the characteristic that tumours behave as acutely-responding tissues and surrounding normal tissues as late-responding. Under these conditions, the difference in α/β ratios results in the selective kill of tumour cells and sparing of normal cells by repeated doses of radiation (Peters and Withers, 1982; Withers *et al.*, 1982). This phenomenon is based on the assumption that normal cells are able to repair radiation damage more effectively than tumour cells (Hall, 1994). However, previous studies have shown that radioresistant tumours may behave like late-responding rather than acutely-responding tissues, rendering further radiotherapy as ineffective and potentially detrimental to the patient (Haas-Kogan *et al.*, 1999; Peters *et al.*, 1982).

1.1.2 *Clinical and in vitro evidence of radioresistance*

In 1956, Puck and Marcus were the first to publish a radiation survival curve for mammalian cells in culture which was the first study characterizing human cervical

cancer (HeLa S3) cell survival as the ability of cells to procreate and form colonies (Puck *et al.*, 1956). Analysis and characterization of radioresistance was first published by Fertil and Malaise, with the conclusion that tumours more resistant to cure by radiotherapy produced cell lines that were more resistant to clinically relevant doses of radiation (Deacon *et al.*, 1984; Fertil and Malaise, 1981). Since then, several reports have established the concept of measuring intrinsic radiosensitivity as a parameter of predicting radiation responsiveness in the clinical situation. Current radiobiological principles dictate that all cell types and tumours can be eradicated by irradiation depending on the dose administered; however, in the clinical situation the dose delivered to the normal tissue surrounding the tumour determines the maximal tolerable dose (Borgmann *et al.*, 2002). Therefore, a better understanding of the mechanisms governing intrinsic radiosensitivity could provide ways to specifically enhance tumour radiosensitivity which would be of great clinical impact since it would minimize normal tissue damage and allow for a more efficient treatment of the tumour tissue.

1.2 THE RADIORESISTANT AND RADIOSENSITIVE PHENOTYPE

1.2.1 *The cellular response to ionizing radiation*

IR-induced cell death induced by genomic damage is generally divided into cell cycle-independent death (pre-mitotic/interphase) and mitotic cell death (reproductive/clonogenic). In both cases, morphological features of cell death can

resemble apoptosis, also known as programmed cell death (Hall, 2000a), and are associated with changes including nuclear condensation of the chromatin, DNA fragmentation, cytoplasmic membrane blebbing, externalized phosphatidylserine residues, and cell shrinkage (Loo and Rillema, 1998; Shiratsuchi *et al.*, 2002; van Heerde *et al.*, 2000). In contrast to this, cell death in necrotic cells occurs due to an influx of fluid through holes in the membrane which causes cellular swelling and lysis (Dive *et al.*, 1992).

The main intrinsic factors governing radiosensitivity include the cell's ability to detect and repair DNA damage and the activation of cellular responses such as cell cycle arrest and induction of apoptosis. The response to IR is also cell type dependent which is demonstrated by a study which shows that thymocytes, splenocytes, hematopoietic progenitor cells (Zhivotovsky *et al.*, 1993) and intestinal crypt cells (Roberts *et al.*, 2003) undergo apoptosis in response to IR and are the most radiosensitive, while fibroblasts undergo irreversible growth arrest and are the most radioresistant (DeSimone *et al.*, 2000). IR-induced apoptosis is not common in other cell types than the ones mentioned above (Allan, 1992).

IR can also induce cell death by mitotic catastrophes observed in many solid tumours (Hendry and West, 1997) where cells fail to undergo successful division due to persistent DNA damage, resulting in giant, multi-nucleated cells. Mitotic catastrophes may in turn result in either necrosis or apoptosis (Ianzini and Mackey, 1998).

One of the first discovered molecular events distinct for the apoptotic changes in morphology was the cleavage of DNA into distinct fragments of equal length. The visualization of these 185 base pair DNA fragments during agarose gel electrophoresis gave rise to the term "DNA ladder" since the dispersal pattern of these low molecular weight DNA fragments within the gel matrix resembled the rungs of a ladder. A Ca2+/Mg2+ -dependent endonuclease (i.e. deoxyribonuclease I - Dnase I) or a caspase-activated deoxyribonuclease (CAD) has been shown to be responsible for the this controlled degradation of genomic DNA by apoptosis (Boone *et al.*, 1995) (Sakahira *et al.*, 1998). The emergence of DNA ladders in electrophoretic gels was once thought of as a hallmark of apoptosis however, programmed cell death has been shown to occur with the absence of DNA laddering. Because of this, it was theorized that DNA laddering may be a consequence of apoptosis, rather than a mediating event (Ormerod *et al.*, 1994; Saraste and Pulkki, 2000). The effect of IR on the degradation of DNA by sufficient doses of radiation is distinct from DNA laddering in that radiation is deposited randomly throughout the DNA chain thus cutting the DNA strand into random sized fragments which may trigger the apoptotic process.

Despite the intricacies involved in IR-induced cell death, some researchers have noted that the induction of apoptosis following a radiation challenge directly causes the radiosensitivity of that cell (Dewey *et al.*, 1995; Fei and El-Deiry, 2003; Garcia-Barros *et al.*, 2003; Olive and Durand, 1997). In contrast, to this a number of studies have also

shown that apoptosis may not be indicative of radiosensitivity since surviving fractions do not reflect the apoptosis index following IR exposure (Wouters *et al.*, 1999; Wouters *et al.*, 1997). Therefore, the sole emphasis on the degree of apoptosis to measure cell viability and radiosensitivity is misleading since cell death involves the loss of its proliferative capacity which is measured by clonogenic assays. Apoptosis is not an accurate measure of total cell death because ionizing radiation causes a number of events not predicted by current apoptotic assays such as prolonged growth by replicative senescence, delayed growth arrest followed by reproductive cell death caused by mitotic catastrophe, and temporary growth delay which may be followed by additional apoptosis or necrosis (Gewirtz, 2000).

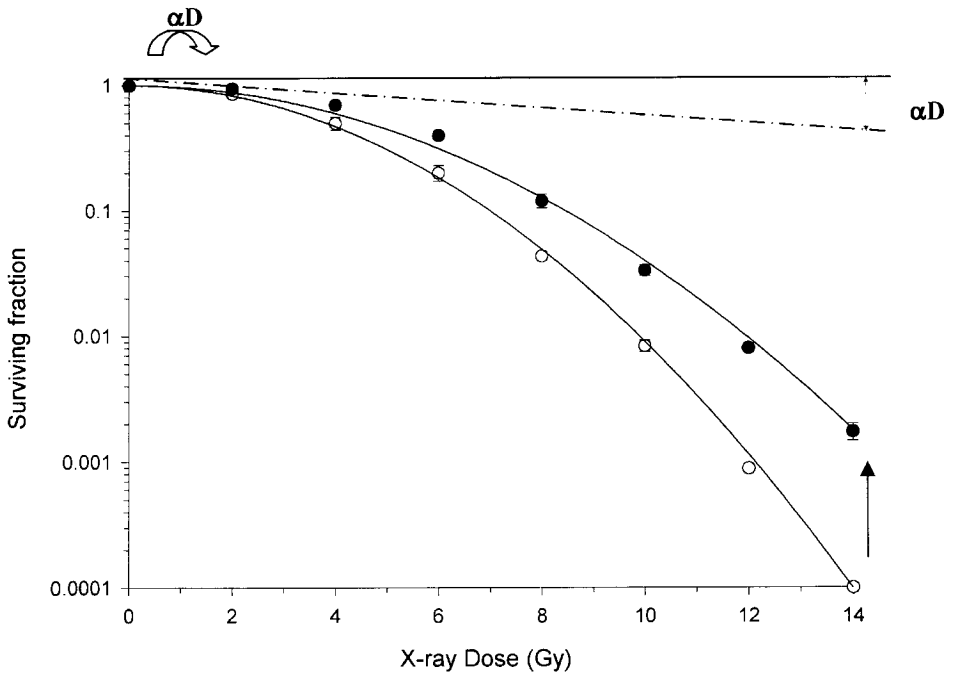
1.2.2 *The clonogenic response to ionizing radiation*

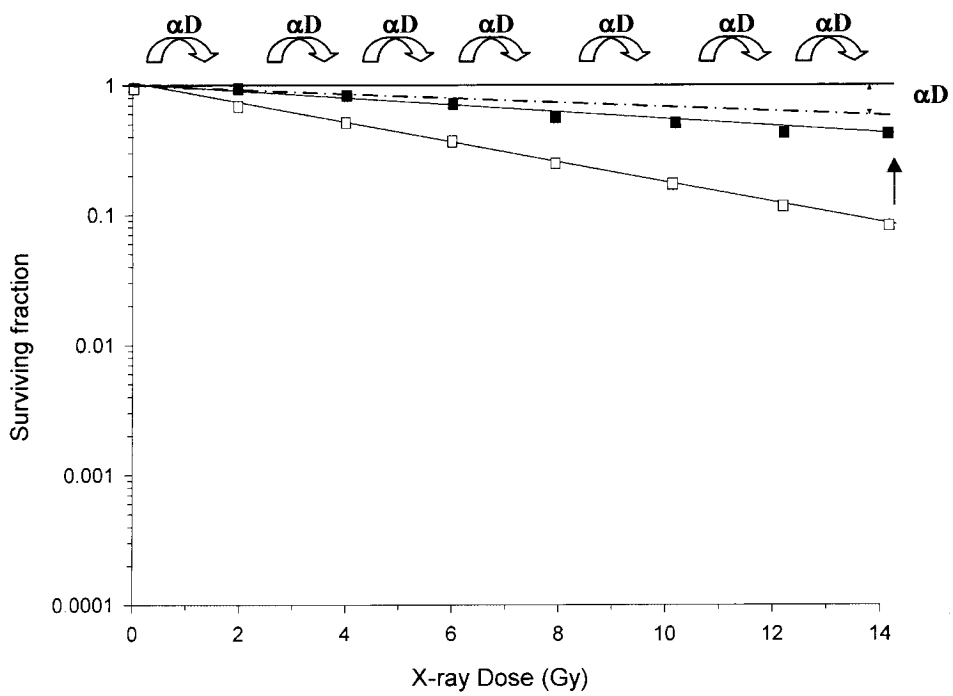
Biochemical markers may not be indicative of the overall biological response to exposure to radiation. The current "gold-standard" for the characterization of radiosensitivity is by measuring the clonogenic potential of a cell type by means of the clonogenic assay (Hall, 2000a). Enhanced clonogenic survival within a given cell type following exposure to X-radiation is indicative of radioresistance which is indicated by an increase in the shoulder of the survival curve (Alaoui-Jamali *et al.*, 1992; Soulieres *et al.*, 1995). This difference in the α-component is reflected in the total shape of the survival curve resulting in an increase in the final slope and can be clearly seen in the diagram illustrating both acute and fractionated survival curves (figure 3.). The α-

component of the survival curve for fractionated radiotherapy is repeated for each fraction of radiation dose administered since a portion of DNA repair is allowed to occur between fractions for a given nontreatment period (i.e. 24 h). This gives the resulting surviving curve a linear appearance. Therefore, a radioresistant cell line will have an increase in the shoulder of the acute survival curve and a decrease in the overall slope of a fractionated survival curve as compared to the radiosensitive (or non-radioresistant) cell line. This phenomenon was demonstrated in isogenic derivatives of glioblastoma cells differing only in p53 function. Glioblastomas containing a dominant negative (dominant because it overrides the wild type activity and is negative because it inhibits the wild type activity) mutant of p53 were shown to have survival curves with significantly lower alpha/beta values at doses less than 4 Gy than glioblastomas containing wild-type p53. Similarly, a daily regimen of 2 Gy for a total of 16 Gy demonstrated an enhanced cytotoxicity for the mutant p53 glioblastoma cell line relative to the parental glioblastoma cell line (Haas-Kogan *et al.*, 1999). Additional findings provided evidence that a decrease in radiation-induced apoptosis could not account for the enhanced resistance to fractionated radiotherapy of cells devoid of p53 function and that giving fewer number of fractions of therapy with increased doses per fraction can be therapeutically advantageous for cells possessing this type of mutation (Haas-Kogan *et al.*, 1999). Unfortunately, most published reports examine radioresistance in the context of single, acute doses of irradiation and so the significance of measuring fractionated radioresistance, relevant in the clinical setting, has not been fully realized. Therefore, measurement of radioresistance within a fractionation scheme can provide us a more accurate reflection of how a cancer

cell may behave in a clinical environment and possibly be a predictive measure in the radiocurability of a particular cancer cell type.

Figure 3. Representation of acute and fractionated survival curves of a radioresistant and a radiosensitive cell line. Circles represent survival curves of cells exposed to acute or single doses of IR. Square symbols correspond to cells exposed to fractionated or split doses of X-radiation (i.e. 2 Gy per day). For fractionated doses of X-radiation, the X-axis displays the total accumulated dose of X-radiation. Open circles and squares represent the survival curves of a radiosensitive cell line. Closed symbols reflect survival curves of a radioresistant cell line. The survival curve for fractionated radiotherapy is linear in appearance since DNA repair is allowed to occur between fractions thus repeating the (αD) linear component of cell kill (bottom panel) where D is the dose of radiation and α is a constant which is dependant on the repair capacity of double strand breaks produced by a single ionizing event.


αD
αD
1
0.1
0.01
0.001
0.0001
Surviving fraction
0
2
4
6
8
10
12
14
X-ray Dose (Gy)


αD
αD
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αD
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αD
1
0.1
0.01
0.001
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Surviving fraction
0
2
4
6
8
10
12
14
X-ray Dose (Gy)

1.3 FACTORS THAT AFFECT RADIORESPONSE

1.3.1 *Hypoxia and the radioresponse*

Solid tumour hypoxia has long been recognized as an impediment in the eradication of cancer by radiotherapy. Hypoxia or a depletion of oxygen in the cells and tissues is known to cause resistance to IR since oxygen is required for IR to generate DNA damaging free radicals (Hall, 2000c). These hypoxic areas are often found within a solid tumour because of poor vascularization, increased diffusion distances and anemia by tumour-associated or therapy-induced factors, all of which impairs the delivery of oxygenated blood to the inner regions of the tumour (Vaupel *et al.*, 2001). Current methods for overcoming tumour hypoxia are to increase the delivery of oxygen, promote angiogenesis and increase vascular permeability. Increasing the delivery of oxygen often involves therapies such as hyperbaric oxygen breathing, blood transfusions, hemoglobin-oxygen affinity modifiers, the administration of carbogen and nicotinamide, or the delivery of chemical radiosensitizers such as oxygen-mimetic drugs (Greco *et al.*, 2003; Marples *et al.*, 2003a; Overgaard and Horsman, 1996). In addition, a study by Denekamp et al showed that increasing the number of fractions of X-radiation at 0.5 Gy per fraction may be more effective than oxygen mimetic chemical sensitizers and may overcome this resistance to IR (Denekamp *et al.*, 1998). Hypoxic radioprotection is not only based on the tumour environment but can also be an intrinsic factor. For example, high basal protein levels of the transcription factor subunit hypoxia-inducible factor-

1alpha (HIF-1alpha), known to affect the hypoxic response of cells, have been associated to poor outcome in several tumour types, and has been suggested as a potential endogenous marker of tumour radioresistance (Vordermark and Brown, 2003). Similarly, another example of an intrinsic factor involving inherited radiosensitivity is shown in the SW620IR1 cell line, derived from irradiated SW620 human colon cells, which has repressed basal antioxidant activities resulting in increased and prolonged oxidative stress following radiation exposure (Tulard *et al.*, 2003).

1.3.2 *DNA damage and their correlation to radiosensitivity*

Radiosensitivity of cells varies during the cell cycle (Sinclair, 1968), likely because the changes in the structure of the DNA that takes place in different cell cycle phases alters the accessibility of DNA repair enzymes. Cells are normally the most radioresistant in S-phase (Marples *et al.*, 2003b) when high-fidelity homologous repair is active and the intra S and G2/M checkpoints should ensure the detection of erroneous repair contributing to radioresistance (Lisby *et al.*, 2003). In contrast, radiosensitivity is increased during the mitotic phase when the chromatin is put under mechanical stress as it is compacted into chromosomes, possibly causing more complex DSBs (Woudstra *et al.*, 1996b) which the DNA repair machinery has little time to act on before the chromosome segregation.

X- and γ-ray radiations produce the majority of DNA breaks in the form of single strand breaks (SSBs) (Hall, 2000b). SSBs are thought to occur frequently in the cell due to replication, transcription and metabolic production of reactive oxygen species, and can be quickly and efficiently dealt with in the cell by utilizing components of the base-excision repair system (Hoeijmakers, 2001). However, SSBs can lead to cell damage and death if repair mechanisms are unable to compensate due to a high number of breaks. The DNA double-strand break (DSB) is considered the most deleterious DNA damage and the frequency of induced DSBs directly correlates with the manifestation of chromosomal aberrations and loss of clonogenicity (Iliakis, 1991; Iliakis *et al.*, 1991; Radford and Broadhurst, 1986). Detection and correct repair of DSBs is of outmost importance for the survival of the cell, conserving the genome's integrity and avoiding carcinogenesis.

Several investigators have shown that there was significantly increased radiosensitivity in various mammalian cell lines which had deficient DSB repair compared to those which had normal dsb repair (Coleman and Stevenson, 1996; Fukushima *et al.*, 2001; Maity *et al.*, 1997; Powell and Abraham, 1993a). *In vivo* evidence of this comes from patients with the autosomal recessive disorder, ataxia telangiectasia (AT) characterized by extreme radiosensitivity and a several hundred fold greater incidence (from normal) for the development of lymphoma due to a mutation in the ataxia telangiectasia mutated (ATM) gene (Meyn, 1995). ATM and ATM-Rad3-related (ATR) genes belong to the phosphoinositide 3-kinase-related kinase (PI3K) gene superfamily, with their translated products functioning as proximal DNA damage-

signaling kinases that regulate diverse cellular processes including cell cycle checkpoint activation, DNA repair, gene transcription, and apoptosis. Therefore, the ATM gene is known to play a role in the regulation of the radioresponsiveness of a cell to IR and other genotoxic or radiomimetic agents that induce DNA double-strand breaks (DSBs) (Bao *et al.*, 2001; Falck *et al.*, 2001; Tibbetts *et al.*, 2000). One particular study claimed that DNA SSB induction and repair was not an important aspect in the radiation response of human tumour and normal cell lines and that the critical factor for underlying radioresistance was the rate at which DNA DSBs were repaired (Schwartz and Vaughan, 1989).

Even though most *in vitro* studies declare a positive correlation between unrepaired DSBs and cell death (Cassoni *et al.*, 1992; El-Awady *et al.*, 2003; Kelland *et al.*, 1988; Kiltie *et al.*, 1997; Sak *et al.*, 2002) a few others have reported no correlation (Johnston *et al.*, 1997; McMillan *et al.*, 1990; Woudstra *et al.*, 1996a).

1.3.3 *p53 status and the radioresponse*

A key molecule in the cellular response to DNA damage is the tumour suppressor p53 for which activation of p53 can cause cell cycle arrest, or trigger apoptosis. The p53 protein was first discovered in 1979 and was characterized as a 53-kDa nuclear phosphoprotein consisting of 393 amino acids (Lane and Crawford, 1979; Linzer *et al.*, 1979; Soussi and May, 1996). Upon DNA damage, p53 can be phosphorylated via the

ATM-Chk2 pathway at serine 15, or by DNA-PKcs at serine 15 and 37 (Shieh *et al.*, 1997; Tominaga *et al.*, 1999) which inhibits binding with its regulator Mdm-2, resulting in p53 accumulation. Nonphosphorylated p53 associates with Mdm-2 which transports p53 from the nucleus and targets it for proteasomal degradation (Chen *et al.*, 1995). Increased expression of p53 also leads to transcriptional upregulation of Mdm-2 causing a negative feedback loop which regulates the level of p53 (Wu *et al.*, 1993). Mdm-2 can also be phosphorylated by ATM or DNA-PK which prevents its binding to p53 (Mayo *et al.*, 1997; Shieh *et al.*, 2000). p53 has many diverse functions (Vousden and Lu, 2002) being a stimulatory transcription factor for the expression of genes encoding pro-apoptotic proteins, e.g. Fas, TNF, TRAIL, Bax, Puma and Noxa, while at the same time a repressor for expression of anti-apoptotic proteins such as B-cell lymphoma-2 (Bcl-2) and inhibitors of apoptotic proteases (IAPs). Moreover, p53 induces cell cycle arrest via upregulation of the cyclin-dependent kinase inhibitor p21/WAF1/CIP1.

It is not exactly understood how the p53 response is directed towards cell cycle arrest, repair and survival, or induction of apoptosis. Two theories have been proposed for the direction of p53 activity (Vousden, 2000). The first theory signifies that it is the availability of regulatory factors present in the cell that control p53 function with the expression of regulatory factors being dependent on the cell type, stage of differentiation and phase in the cell cycle. For example, the presence or absence of growth factors can influence p53-mediated growth arrest or apoptosis. One example being murine bone marrow-derived cells which are resistant to IR-apoptosis when cultured in the presence of growth factors such as interleukin (IL)-3,

IL-4, and Insulin-like growth factor (IGF-1) (Collins *et al.*, 1992). In addition, mouse melanoma cells expressing antisense-IGF-1 receptor transcripts, used to downregulate the IGF1 receptor protein, were found to be more radiosensitive *in vitro* and *in vivo* than controls, and had a reduction in DNA synthesis with decreased nuclear accumulation of radiation-induced wild-type p53 and reduced levels of phosphorylated p53 serine 18, an equivalent of serine 15 in the human known to be critical in DNA damage signalling (Macaulay *et al.*, 2001). The second theory is that levels and modifications of p53 are responsible for the differential expression of genes. Activation and selection of the type of DNA repair by p53 appears to rely on specific modifications of its amino acid residues. One modification, acetylation of p53 at lysine residues, can occur late after IR inducing DSB DNA repair, or early following UV radiation inducing nucleotide excision repair (Dumaz and Meek, 1999; Dumaz *et al.*, 1999). Similarily, phosphorylation of p53 plays an important role not only in its stability but also in its mode of action. For example, the phosphorylation of p53 at its C terminus by cdk, protein kinase C, or casein kinase II stimulates the sequence specific DNA binding ability of p53 *in vitro*, thus affecting its DNA damage repair triggering capability (Ko *et al.*, 1997).

Genotoxic damage, induced by either chemotherapy or radiotherapy, induces p53 overexpression in order to control the rate of proliferating damaged cells, thus triggering the DNA repair or apoptosis (Ricevuto *et al.*, 2003). In many tumour types, wild type p53 function is abrogated by mutations thereby generating nonfunctional and more stable p53

proteins that accumulate to very high levels in the nucleus of tumour cells and are associated with chemo/radioresistance due to the inability to initiate cell death and maintain genetic integrity (Couture *et al.*, 2002). For example, tumours devoid of the p53 gene, when injected into immunocompromised mice, are more resistant to gamma radiation and contain fewer apoptotic cells compared with tumours that express the wild type p53 gene (Lowe *et al.*, 1994). Another investigative study demonstrated that the radioresistant head and neck carcinoma cell line (JSQ-3) containing a mutant form of p53 was suppressed *in vitro* and *in vivo* by treatment of the cells with a viral vector containing wild type p53 (Pirollo *et al.*, 1997). A correlation of the nuclear accumulation and the abnormal overexpression of p53 with the failure of local treatment in patients with squamous cell carcinoma of the head and neck region suggested that these markers may assist in predicting radioresistance (Couture *et al.*, 2002).

Even though evidence has clearly established that p53 activity is a potent pro-apoptotic stimulus, it is not necessary for IR-induced apoptosis as cell types absent in functional p53 have been shown to experience apoptosis and cell death in response to radiation (Bracey *et al.*, 1995; Haas-Kogan *et al.*, 1996; Kyprianou and Rock, 1998; Unruh *et al.*, 2003; Wouters *et al.*, 1999). For example, human prostate cancer cells (PC-3) transfected with the plasmid encoding the mutant p53 sequence were radiosensitive and continued to show apoptosis in response to ionizing irradiation thus illustrating that the introduced mutations in these cells provided no enhancement in the development of radioresistance (Kyprianou and Rock, 1998). Additionally, malignant human lymphoma

(DL-40) cells containing mutant p53 experienced IR-induced apoptosis mediated by a p53-independent signaling pathway. However, closely-related cell lines with wild-type p53 (DL-95 and DL-110) had a significantly delayed onset of cell death for which the mechanism of inhibition was unknown (Ogawa *et al.*, 2000). Interestingly, tumours formed in nude mice from HCT116 colorectal cells abrogated of p53 were shown to have considerably more radiation-induced apoptosis but were more radioresistant than tumours with wild-type p53. Further analysis of HCT116 cells devoid of p53 *in vitro* had also shown enhanced radiation-induced apoptosis correlated with an increase in clonogenic radioresistance which was attributed to an increase in the number of radioresistant S-phase cells. This mechanism was shown to be due to lack of induction of the cyclin-dependent kinase inhibitor p21/WAF1/CIP1 by p53 and *in vivo* analysis demonstrated a unique role for the radioprotectiveness of p21/WAF1/CIP1 independent of p53 (Wouters *et al.*, 1999). Therefore, activation of p53 in tumours can act as a radiosensitizer but in over 50% of cases, tumours have mutant p53 (Lain and Lane, 2003) and vary amongst each other in radiosensitivity independent of p53 (Schafer *et al.*, 2002). Clearly, the effect of p53 on cellular radiosensitivity is dependent on the particular cell type and is one of multiple factors that may determine the radioresponsiveness of cells.

1.3.4 *IR-induced cell cycle perturbations and radioresistance*

Cell proliferation is known to be delayed in response to DNA damage through alterations in cell cycle induced by a variety of stimuli including IR (Kastan *et al.*, 1991).

Arrests in the cell cycle are controlled by a number of "checkpoints", for which cell cycle dependent kinases (cdks) are responsible (Hartwell and Weinert, 1989; Kaufmann and Paules, 1996), and can occur following DNA damage in the G1 or G2 phases (O'Connor, 1997). For example, cells from AT patients are known to be exquisitely sensitive to IR and also display defects in the IR-induced G1/S, intra-S, and G2/M cell cycle checkpoints (Falck *et al.*, 2001; Kastan *et al.*, 1992). Therefore, the purpose of cell cycle arrest would be to allow the cell to repair DNA damage before progression into S phase or mitosis as DNA-damaged cells, if allowed to proliferate without repairing the damage, have been shown to produce mitotic catastrophes (O'Connor, 1997).

It was suggested that increased radioresistance correlates with a prolonged G1 arrest following DNA damage (Marples *et al.*, 2003b; Yau *et al.*, 1980) with the rationale in this theory, being that the longer a cell spends in the G1 phase, the more time is granted for DNA repair before the onset of DNA replication. Inconsistent with this is previous evidence showing that a prolonged G1 cell-cycle phase does not necessarily produce radioresistance (Haas-Kogan *et al.*, 1996; Hill *et al.*, 1999; Siles *et al.*, 1996); the reason being that the activation of tumour suppressor p53 by IR not only modulates the activity of the G1 checkpoint, but also the initiation of apoptosis.

Interestingly, activation of p53 does not affect G2 arrest and the ability to arrest in this phase of the cell cycle is present in most tumour cells (O'Connor, 1997). Cells which are deficient in G2 arrest replicate abnormally with missing or inappropriate numbers of

genes or chromosomes, demonstrating that G2 arrest is important for cell survival (Powell *et al.*, 1995; Russell *et al.*, 1995; Tsuboi *et al.*, 1997). The ATM-Rad3-related (ATR) gene, mentioned previously, has been shown to be required for G2/M checkpoint function in response to IR and performs at least two distinct S phase checkpoint tasks. ATR is first required for the DNA replication checkpoint, which delays the onset of mitosis in the presence of unreplicated DNA, while subsequently required to prevent the collapse of the replication fork and DNA strand breakage when DNA replication is momentarily subdued. It is no surprise then that when mutations are introduced in the ATR gene, similar to what is seen in cells from AT patients, increased radiosensitivity to x-rays and a loss of checkpoint control are exhibited independent of wild-type p53 (Cliby *et al.*, 1998; Wright *et al.*, 1998).

1.4 RADIORESISTANCE: INDUCED OR INHERENT?

It is known that fractionated x-radiation given *in vitro*, can produce subpopulations of tumour cells that are more resistant than the original cell line (Li *et al.*, 2001). However, the debate still continues as to whether this radioresistant phenotype is intrinsic or acquired in response to radiation therapy. In other words, the two possible explanations for the presence of the radioresistant cells is that the fractionated treatment had selected for tumour cells that were more resistant or that the treatment had caused an inducible radiation response somewhat akin to the adaptive response. An adaptive response to radiotherapy is of grave concern since fractionated doses or a very low dose

rate of IR could result in radiation-induced resistance in a clinical setting through the introduction of genetic mutations.

In radiobiology, the "adaptive response" is defined as the induction of cellular resistance to radiation damage and is often described in the context in which cells exposed to a low priming dose of radiation are less susceptible to DNA damage by a subsequent high dose of radiation. The adaptive response to small radiation doses has been studied for many years, particularly in human lymphocytes. In these studies, exposure to a low dose of radiation prior to a high dose reduced the number of chromosomal aberrations as compared to a high dose without a priming low dose (Olivieri *et al.*, 1984; Wiencke *et al.*, 1986). Some studies have shown that such priming doses can increase cell survival against a challenge dose, even at priming doses as high as 2 Gy (Azzam *et al.*, 1994; Raaphorst and Boyden, 1999; Shadley and Dai, 1992). It is hypothesized that the adaptive response may be a result of either a radiation-induced DNA damage-sensing threshold that triggers faster, more efficient DNA repair or may be due to changes in the organization or structure of DNA that facilitates constitutive repair (Olivieri *et al.*, 1984; Wolff, 1992; Wolff, 1998; Wouters and Skarsgard, 1997).

Another closely related phenomenon that has been originally reported by Marples and Joiner is distinct from the adaptive response and exhibits a region of low-dose hypersensitivity (usually below 0.5 Gy), followed by an increase in radioresistance as a function of dose until about 1 Gy (Joiner *et al.*, 2001; Lambin *et al.*, 1996; Marples and

Joiner, 1993; Skarsgard and Wouters, 1997). Interestingly, both low dose hypersensitivity and induced radiation resistance are most prevalent in radioresistant cell lines, since several radiosensitive cell lines did not show radioresistance beyond 0.5 Gy (Lambin *et al.*, 1996; Wouters *et al.*, 1996). One plausible explanation for the occurrence of low dose hypersensitivity is that doses below 0.5 Gy is insufficient DNA damage to trigger repair systems or other radioprotective mechanisms (Lambin *et al.*, 1996; Marples *et al.*, 2004; Wouters *et al.*, 1997).

The adaptive response and the low-dose hypersensitivity followed by single dose induced radioresistance may have similar mechanisms since it was shown that low dose hypersensitivity disappeared if cells have been previously exposed to a low adaptive dose of radiation prior to the challenge dose (Joiner *et al.*, 1996; Marples and Joiner, 1995). Further studies have shown that DNA repair processes are likely to be involved in the development of increased radioresistance (Joiner *et al.*, 2001; Skov, 1999). Evidence of this was shown with the application of 3-Aminobenzamide, a potent inhibitor of the DNA repair enzyme poly(ADP ribose)-polymerase (PARP), which prevented the development of single dose induced radioresistance and the adaptive response (Kleczkowska and Althaus, 1996; Marples and Joiner, 2000). However, there are significant differences between the adaptive response and the single dose induced radioresistance. Several distinct differences are that single dose induced radioresistance is not affected by changes in cell cycle during treatment or changes in levels of intracellular factors that protect against immediate DNA damage whereas the adaptive response is sensitive to these

factors. Also the DNA repair mechanisms responsible for the adaptive response and for the single dose induced radioresistance may be different. For example, the radioadaptive response induced by fractionated irradiation of small-cell lung cancer (H69) cells was found to be correlated with increased expression of multidrug resistance-associated proteins MRP 1, MRP2, and topoisomerase II alpha expression; a nuclear enzyme involved in chromosome condensation, chromatid separation, and the release of torsional stress that occurs during DNA transcription and replication (Boege, 1996; Henness *et al.*, 2002). In contrast, another study had demonstrated that novobiocin, an inhibitor of topoisomerase II, failed to inhibit the development of single dose induced radioresistance in Chinese hamster (V79-379A) cells (Marples and Joiner, 2000).

Intrinsic cellular resistance to radiation is known to vary from cell type to cell type (Deschavanne and Fertil, 1996) and even within so-called "homogeneous" cell lines (Frykholm *et al.*, 1991; Leith *et al.*, 1984; Leith, 1982). Further evidence for inherent radioresistance within a cell line was derived from several subclones isolated from the nonirradiated parental (MDA-MB231) breast cancer cells upon analysis of the radioresponsiveness of the cells to fractionated radiation. Clonogenic survival assays showed that three to five fractions of 2-6 Gy per IR fraction resulted in the selection of radioresistant subpopulations. While, the mechanism responsible for the selection of radioresistant cells was not addressed, the changes in cell cycle checkpoints and growth rates were similar for both the isolated radioresistant subclones and the nonirradiated parental population. Further irradiation of the radioresistant subclones did not increase

radioresistance giving some evidence of selection rather than adaptation to the IR treatment (Pearce *et al.*, 2001). Conversely, in an earlier study, two squamous cell carcinoma lines determined inherently sensitive to single doses of IR showed enhanced survival after fractionated radiation, whereas the radiosensitivity remained the same, after fractionated treatment, in cell lines that were radioresistant to single dose of IR. These results suggested that cell populations are heterogeneous containing both cancer cells which are inherently resistant and those which are adaptive to irradiation. This study also gives no indication that radioadaptability is related to the cell's intrinsic radiosensitivity. The appearance of adaptive cells may possibly be accredited to the repair of sublethal radiation damage and as such, shortening of the total irradiation time may prevent cancer cell recovery (between fractions) in some, but not in all carcinomas (Pekkola-Heino *et al.*, 1991).

Only recently have studies examined the role of potential oncogenes, growth factors, or secondary messengers on intrinsic radioresistance. Some cell types containing enhanced basal signaling by proto-oncogenes, such as retrovirus-associated DNA sequences (RAS), have been shown to be radioresistant. RAS genes, originally isolated from harvey (h-ras) and kirsten (k-ras) murine sarcoma viruses, encode for small transforming GTP-binding proteins that play a critical role in normal cellular growth, differentiation, and development, and when mutated have the potential for causing malignant transformation. The ability of the ras oncogene to lead to radioresistance has been demonstrated by a number of independent studies which have shown that

overexpression of ras lead to malignant transformation of rodent or human cells, resulting in cell lines that are substantially more resistant to radiation than the parental cells (Ling and Endlich, 1989; McKenna *et al.*, 1990; Miller *et al.*, 1993). In addition, inhibition of oncogenic ras activity, by the ras inhibitor FTI-277, lead to radiosensitization of radioresistant rat embryo cells transfected with h-ras (Bernhard *et al.*, 1996), and in human tumour xenografts with activated h-ras gene (Cohen-Jonathan *et al.*, 2000). The more specific ras inhibitor R115777 only had a radiosensitizer effect for radioresistant human glioma cell lines and did not affect survival of radiosensitive cells and it was suggested that R1157777 might selectively target radioresistant gliomas without affecting normal adjacent tissue (Delmas *et al.*, 2002). Finally, human tumour (HT1080) cells comprising of activated endogenous ras were found to have higher clonogenic survival to radiation than in cells not containing the mutant-activated ras which provides additional evidence that intrinsic radioresistance in tumour cells is reduced after loss of ras functionality (Bernhard *et al.*, 2000).

The activation of ras, whether by mutation or by cell-surface receptor activation, is known to trigger signaling pathways through the activation of a number of cytoplasmic kinases such as mitogen activated protein kinase (MAPK) (Grana *et al.*, 2002) and phosphatidyl-inositol-3'-kinase (PI3K), (Rodriguez-Viciana *et al.*, 1994) and it is the identification of these ras signaling pathways leading to radiation resistance which is of considerable interest.

Numerous cell types, show radioprotection through PI3K pathway, whereas some other cell types are made more radioresistant by activation of either the transcription factors nuclear factor kappa beta (NFKB), or MAPK. Growth factor receptors, such as the epidermal growth factor (EGF) receptor, can be induced by IR. This demonstrates the activation of a number of downstream pathways, such as the serine/threonine protein kinase (AKT) pathway (also known as the protein kinase B pathway), and the MAPK pathway, leading to radioprotection. The application of kinase inhibitors that inhibit signaling by EGFR have been shown to potentiate radiosensitization (Sartor, 2003). Therefore, a greater number of these receptors that are present on the cell surface or an increase in receptor activity may provide a greater radioprotective effect (Dent *et al.*, 2003; Liang *et al.*, 2003; Sartor, 2003).

Further investigations are required in untreated cells prior to irradiation in order to determine the degree of radioresistance that can be attributed to genetic predisposition versus adaptive ability.

1.5 RADIORESISTANCE AND CHEMOTHERAPY

A variety of chemotherapeutic drugs have been employed in the treatment of cancer and against recurring tumours these include the classical radiosensitizing agents, halogenated pyrimidines and nitroimidazoles and some conventional cytotoxic agents, such as hydroxyurea, 5-fluorouracil (5-FU) and cisplatin. Hydroxyurea has also been

used because of its ability to inhibit excision-repair of thymine dimers and single-strand DNA breaks induced by radiation (Schilsky, 1992).

Camptothecin (CPT) analogs, designed in the 1990s, are well known drugs that are used in the treatment of human cancer. CPT was initially derived from the chinese tree, Camptotheca acuminata, but has also been found in the nothapodytes tree, Nothapodytes foetida. CPT has been shown to specifically inhibit the breakage/rejoining reaction of DNA topoisomerase I (Pommier *et al.*, 1995) but this drug was found to be too toxic for clinical use. The knowledge of the biochemistry of CPT has been vastly increased since its discovery, with the development of the CPT analogs, irinotecan (CPT-11, Camptosar), and topotecan which are more clinically relevant since they are less toxic and more easily dissolved in water for injection. Preclinical studies have shown that these CPT agents are potent radiosensitizers, providing a strong rational for using these drugs in combination with radiotherapy (Milas *et al.*, 2003). Currently, a more recently designed analog of CPT, topotecan (Hycamptin) was shown to be effective and was approved by the Health Canada therapeutic products program (Health-Canada, 2004) in the treatment of both small cell lung carinoma (Ormrod and Spencer, 1999) and epithelial ovarian carcinoma (ten Bokkel Huinink *et al.*, 1997). Although, it has not been approved in the treatment of brain tumours, in a phase II study, it was demonstrated to have potential in treating and preserving the quality of life of patients with glioblastoma multiforme which is known to be a tumour with pronounced radioresistance (Gross *et al.*, 2001).

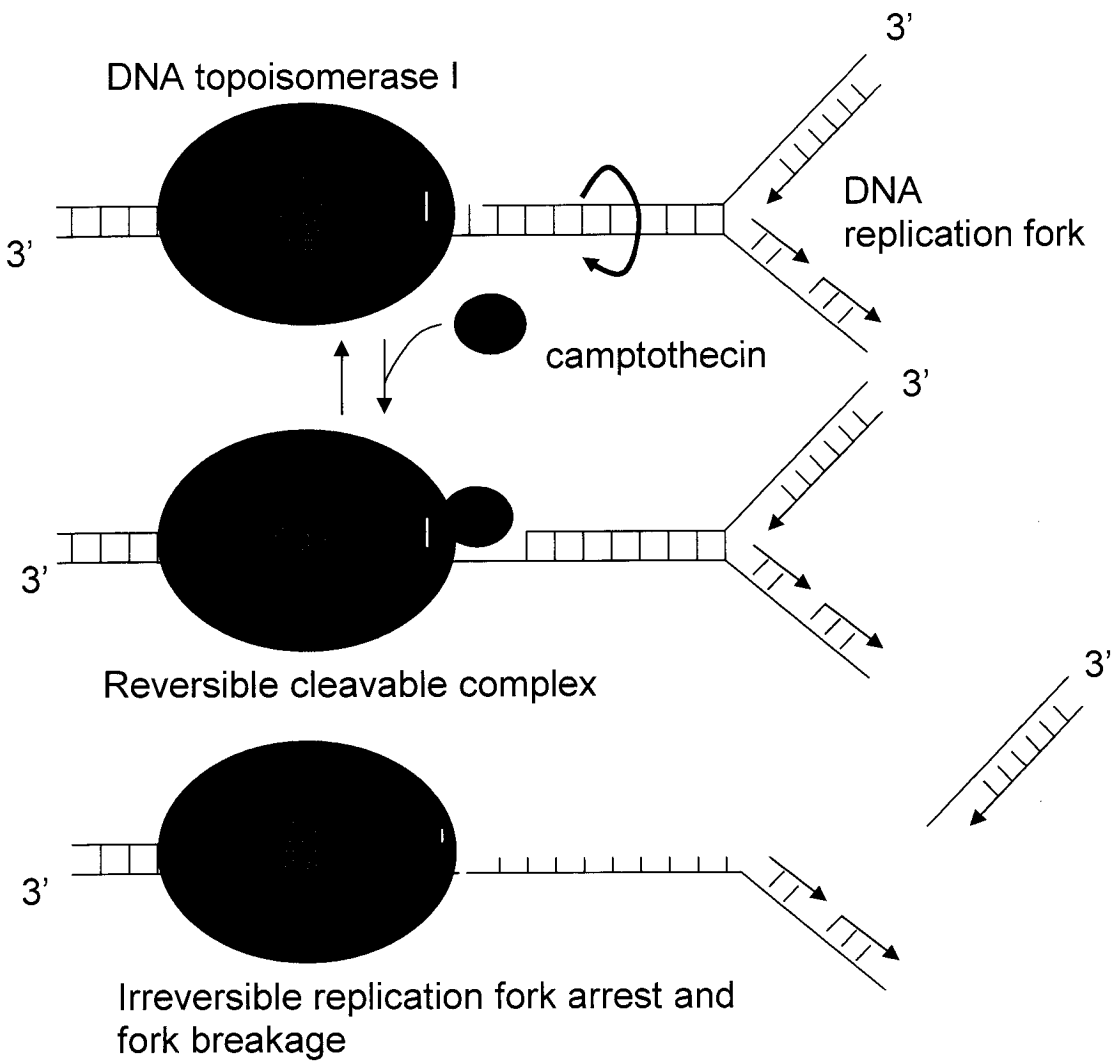
A target of CPT and its analogs is the DNA repair-associated protein DNA topoisomerase I, a monomeric protein of 100 kDa, that relieves torsional strain in DNA by inducing reversible SSB during S-phase. CPT's cytotoxicity is derived from its ability to produce a reversible cleavable complex with topoisomerase I which prevents rejoining of the fragmented DNA strand during DNA replication when it encounters the advancing replication fork and develops into a potentially lethal DSB (Rich and Kirichenko, 1998) (figure 4). As a result, CPT is a potent cell death-inducing agent that is cell cycle specific targeting cells in their S-phase. CPT can also induce cellular differentiation, contributing to its antitumour activity, and has been shown to inhibit DNA and RNA synthesis causing rapid chromosomal fragmentation (Daza *et al.*, 2002; Squires *et al.*, 1991; Suzuki and Kato, 1996). Work from our own laboratory has shown that CPT can also potentiate low dose rate irradiation, and that interestingly, the mechanism of potentiation may be related to an increase in DNA DSB's (Owen *et al.*, 2002). This has been supported by other studies which have also demonstrated that the mechanism of CPT toxicity is primarily due to the generation of DSB's (Ryan *et al.*, 1994; Slichenmyer *et al.*, 1993).

The rationale for combining S-phase agents like CPT analogs with X-radiation is that they target the relatively radioresistant S-phase cells (Dubrez *et al.*, 1995) and for this reason CPT analogs may be proficient in killing rapidly repopulating tumour cells that may arise during fractionated radiotherapy. The effects of the CPT analog 9-aminocamptothecin combined with single and fractionated irradiation on a mouse

mammary cancer was studied and was found to be very effective in minimizing tumour regrowth resulting in radiation sensitization of the cancer. This S-phase inhibitor was found to be better tolerated by the mice when given during the rest phase of the mouse, when intestinal mucosal proliferation is relatively low. This study provides proof that time of administration of this radiosensitizer is equally as important as its dosing in minimizing normal tissue damage (i.e. gastrointestinal tract) and maximizing tumour toxicity (Kirichenko and Rich, 1999). A phase I dose study showed that 75% patients treated for locally advanced head and neck cancer showed a complete response to conventional fractionated radiotherapy consisting of 2 Gy per day for, 5 fractions per week to a total dose of 66-70 Gy, when combined with irinotecan and docetaxel (Koukourakis *et al.*, 1999). Recently, Health Canada (Health_Canada_Therapeutics_Products_Programme, 2001) has approved irinotecan (CPT-11) for both first- and second-line chemotherapy for metastatic colorectal cancer (BC_Cancer_Agency, 2001a; Folprecht and Kohne, 2004; Rothenberg *et al.*, 1996) and topotecan as a second-line treatment for both ovarian cancer (BC_Cancer_Agency, 2001b; Fields and Runowicz, 2003; Herzog, 2002; Takimoto and Arbuck, 1997), and for small lung cancer (BC_Cancer_Agency, 2001b; Brahmer and Ettinger, 1998; Kreienberg and Freund, 1998). In addition, one phase II study, showed that a combination of CPT-11 and cisplatin was also promising in treating extensive-disease small-cell lung cancer (Takigawa *et al.*, 2003). *In vitro* analysis of the impact of topotecan used as either a single agent or in combination with fractionated doses of IR on clonogenic cell survival was evaluated in three glioblastoma cell lines and in normal fibroblasts of the skin and

lung. The resulting data had suggested that combined IR fractionated treatment with topotecan was found to have an antagonist interaction, inducing resistance to the drug, in fibroblasts, but was found to be additive, in cell killing, in glioblastoma cells (Ohneseit *et al.*, 2002). An additional *in vivo* study used nude mice with subcutaneous human lung cancer (H460) cell xenografts that were treated with an orally administered camptothecin derivative, 9-nitrocamptothecin (RFS-2000), and radiation of 2 Gy per day for 5 consecutive days. The drug RFS-2000 was found to enhance the effects of fractionated radiotherapy, with an enhancement factor (EF) of 1.64, and its synergistic mechanism of action was hypothesized to be due to the inhibition of sublethal damage recovery (Amorino *et al.*, 2000). Lastly, topotecan was given with conventional fractionated radiotherapy (given daily for 33 days) to patients with intrinsic pontine glioma of childhood in a phase I study. This study established the maximum tolerated dosage of daily i.v. administered topotecan and provided evidence that this agent is a potent radiosensitizer for childhood brainstem glioma (Sanghavi *et al.*, 2003). Based on these early phase I and II studies the combination of radiotherapy with this class of agents show promise that the drug has beneficial therapeutic properties in combating possible radioresistant cells, warranting further investigations in different cancer types and in the optimal scheduling of administration.

Figure 4. Mechanism of CPT-induced DNA damage. CPT forms a cleavage complex with the topoisomerase I and the DNA strand. A DNA DSB is potentially created when a DNA replication fork collides with the CPT/topoisomerase I/DNA cleavable complex, possibly resulting in cell death if not repaired.



G_2 cell cycle arrest and *CELLULAR DEATH*

1.6 DNA MICROARRAYS AND THEIR APPLICATION TO IDENTIFYING RADIOSENSITIVE AND RADIORESISTANT GENES

DNA microarrays are a relatively new biotechnology that allows the simultaneous measurement of the expression levels of thousands of genes from a biological sample (Brown and Botstein, 1999) which has allowed the focus of genetics to change from the characterization of genes and pathways on a case-by-case basis to that at the genomic level. Several other technologies have also been recently developed for simultaneous measurements and verifications of the expression levels of genes (i.e. quantitative polymerase chain reaction (Q-PCR)) and their gene products (proteome chips). The sequencing of various genomes, along with a surge in high-throughput technologies, as found with the internet based database Basic Local Alignment Search Tool Algorithm (BLAST), has made it possible to obtain data on thousands of genes from a single biological sample or allow the reconstruction of novel genes from a number of submitted partial cDNA gene sequences, also known as expressed sequence tag sites (ESTs) (Mesak *et al.*, 2003), or unfinished high throughput genome sequences (HTGs). ESTs are often used as identifiers of a particular gene, deposited as spots in microarrays or used in locating and mapping genes. Therefore, high-throughput genome-wide typing of genetic markers can be used to map genes associated with complex diseases or biological responses to external agents. As a result, DNA microarrays are an ideal technology for studying the genetic predisposition of inherent radioresistance.

It is well accepted that every somatic and germ cell of an organism contains a copy of its entire genome and individual genes are encoded in the DNA of the genome. The following schematic represents the simplified premise of genetics:

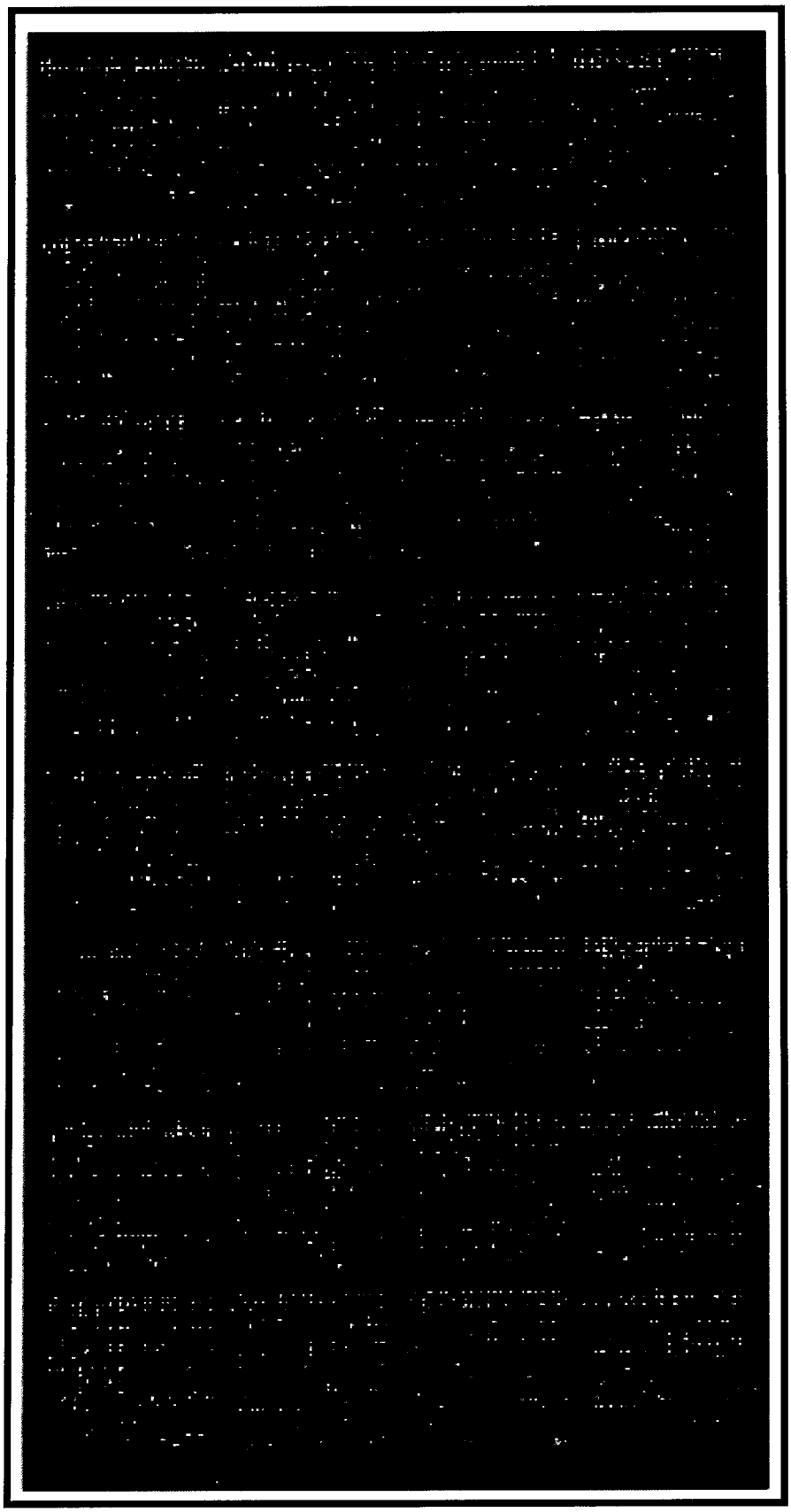
$$\text{DNA} \Rightarrow \textit{transcription} \Rightarrow \text{RNA} \Rightarrow \textit{translation} \Rightarrow \text{Protein}$$

Simply stated, the DNA encoding the gene is transcribed into RNA with the mRNA then being translated into a protein that performs some structural or catalytic function. Based on the role and environment of the cell, this process is carried out at different levels of frequency for each gene. Moreover, genes interact in single or multiple pathways to perform the complicated tasks that keep a cell functioning properly.

The essential feature of the DNA microarray technologies put forth by Schena et al. (1995) (Schena *et al.*, 1995), Velculescu et al. (1995) (Velculescu *et al.*, 1995), and Chee et al. (1996) (Chee *et al.*, 1996) is that they measure the relative abundance of replicated cDNA from mRNA in the sampled cells. Figure 5 demonstrates a cDNA array of about 19,000 human genes. In this type of array, two biological samples are hybridized to the same slide, one of which is usually a reference (control) sample, and the other is the sample of interest. RNA is first extracted from each sample and is then reverse transcribed to the gene's coding cDNA. A green fluorescent dye is attached to the cDNA from one sample, and a red fluorescent dye to the DNA from the other sample. Each spot

on the array is unique and contains cDNA for a specific gene and may be represented multiple times throughout the array. The two samples are then applied to the array, and the cDNA (with dye attached) hybridizes to its complementary strand, at its particular spot. The signal of green and red dyes is measured for each spot giving a crude measurement of the abundance of mRNA present in each sample for each gene and the number reported for each spot is usually the log 2 ratio of red to green signal. The spots are artificially colored from a green to red visible color spectrum according to their comparative spot intensity since the original images from both dyes are in 16 bit grayscale and therefore, in figure 5 green indicates that the mRNA corresponding to the gene at that specific spot is much stronger from the green sample than the red whereas yellow indicates they are about equal, red indicates that the red channel is more abundant than the green, and the absence of color (black) within a spot indicates there is a negligible amount of mRNA present in either sample.

Figure 5. A human cDNA array. Each spot represents a different gene, and the colors represent the relative abundance of the mRNA from the red channel versus the mRNA from the green channel.



One of the earliest uses of microarrays for identifying gene markers of radioresistance was in an apoptosis-sensitive and apoptosis-resistant mouse B cell lymphoma model system both before and after irradiation. Voehringer identified two major gene expression patterns out of 11,000 genes. The first pattern of expression, identified in radioresistant (Lyar) cells as compared to radiosensitive (Lyas) cells before radiation exposure, showed significantly greater levels of expression of genes involved in regulating intracellular redox potential. These genes included the tetraspanin CD53 and fructose-1,6-bisphosphatase; both of which can lead to the increase of the principle intracellular antioxidant, glutathione which has been shown to inhibit many forms of apoptosis. The second pattern of expression was linked to genes that participated in mitochondrial uncoupling and loss of membrane potential. The radiosensitive lymphoma cells had higher expressions of these genes than the radioresistant cells which is not surprising since changes in mitochondrial membrane potential are known to trigger the release of apoptotic factors from the mitochondria causing the activation of the caspase cascade promoting cell death (Voehringer *et al.*, 2000). The interesting points to remember from this is that very few studies have examined radioresistance, prior to radiation treatment, using this methodology and that the cell lines were derived from a parental cell line at one time giving a genetic profile of inherent radioresistance.

Another study acquired, from human patients, biopsy samples from radiation-sensitive and radiation-resistant squamous cell carcinoma of head and neck tumours. Radiation-sensitive tumours, in this study, were defined by the absence of all tumour after 6 weeks of treatment with a total 68–70 Gy, whereas radiation-resistant tumours were defined by a decrease in tumour size of 40%, at the end of the therapy. These tumours were then subjected to gene expression analysis using a cDNA array containing only 1187 tumour-related genes which included oncogenes, tumour suppressor genes, cell cycle genes, and apoptosis genes. They discovered that the genes expressed in the radioresistant and not the radiosensitive tumours included some of the classically known genes responsible for the radioresistant phenotype such as the proto-oncogene and component of the transcription factor AP-1, c-*jun*, and the DNA repair protein, *XRCC1*, but many of the reported changes in gene expression that they have published have not been studied in the context of modulating the radiation responsiveness of cells (Hanna *et al.*, 2001).

Direct irradiation of human lymphoblastiod cell lines with 5 Gy of X-radiation significantly altered expression of 180 of the 6,800 genes on the Affymetrix HuGeneFl GeneChip microarray. With nineteen of these genes being linked to cell cycle (i.e. p21, cyclin G1, and mdm2) and five genes to mitotic machinery. Similar to the previous study, genes involved in DNA repair were identified however these upregulated DNA repair genes (p48, XPC, gadd45, and PCNA) are associated with UV-damage and not IR-induced DNA damage. The authors suggested that it is

possible that these genes may have overlapping roles in DNA repair for both IR- and UV-damage. Lastly, four of the top 34 significantly expressed genes were recognized to play a role in apoptosis and these included the death receptors Fas and TNF-alpha. No role was determined in the remaining significantly expressed genes (Tusher *et al.*, 2001).

Microarray gene expression profiling of yeasts (*Saccharomyces cerevisiae*) possessing homozygous deletions in 3670 different nonessential genes identified 130 yeast strains that showed enhanced γ-radiosensitivity with over 50 % of these yeast genes sharing homology with human genes. Twenty-three of the 130 radiosensitive mutants identified contained genes associated with DNA repair (i.e. RAD52, RAD6 and RAD3) and/or cell cycle checkpoints, including 17 that have been implicated to the development of oncogenesis. Finally, a number of genes were also found to be related to protein metabolism and stabilization and many of the other genes identified were not previously known to provide radioprotection (Bennett *et al.*, 2001).

Birrell et al. attempted to identify genes that are protective against four DNA-damaging agents: ionizing radiation, UV, cisplatin or to hydrogen peroxide in yeast and was unable to isolate genes associated with DNA repair. Very few genes, known to be involved in protecting against DNA damage, were found to be expressed following DNA insult suggesting that DNA damage-inducible gene expression was not necessary for survival and that steady state levels of expression are adequate in

coping with DNA damage from acute exposures. Therefore, it is the levels of pre-existing endogenous proteins involved in protecting against DNA damage that determines the radioresponse, thus supporting the importance of intrinsic radioresistance (Birrell *et al.*, 2002).

In general, all the published studies have evaluated differences in gene expression between radioresistant and radiosensitive established human cell lines, cells derived from primary tumours, or in yeast cells, following exposure to IR (Mercier *et al.*, 2001). These studies have not observed whether genome-wide screening of cell clones with varying radiosensitivities derived from a parental cell line can identify the genes that are important for the expression of the radiation-resistant or radiation-sensitive phenotypes.

1.7 SPECIFIC OBJECTIVES

It is unknown why some cell types are very radiation-resistant. The mechanisms of radioresistance have been attributed to a vast number of intrinsic factors. One factor that has been shown to make a particular cell type radioresistant is the reduced ability to undergo programmed cellular death (Li *et al.*, 2000; Li *et al.*, 1998; Serrone and Hersey, 1999). Also, the types of cell death (apoptosis or necrosis) have been shown to be important in determining the response to treatment with drugs and radiation (Blank *et al.*, 1997; Dewey *et al.*, 1995; Eastman and Rigas, 1999).

Therefore, in order to identify the specific mechanisms of cellular death following concurrent chemoradiation treatment a model system is required that can exhibit resistance or sensitivity to radiation and chemotherapy. Thus the response of this cell type to combined drug and radiation can provide insight from a clinical perspective since it could reveal modes of cell deaths distinct to specific treatments that could be exploited to sensitize radiation therapy and chemotherapy resistant tumours.

One of the limiting factors in the study of radioresistance is that many researchers try to establish radioresponsive relationships between cells from different tissues and cell types. Since, cell heterogeneity can even exist amongst the same tumour tissue, mechanisms involved in radiation sensitivity are unlikely to be distinguished from other intrinsic variable factors. To focus on differences only attributed to radioresistance and radiosensitivity, the use of a model system which differs only by their radioresponsiveness is required. Closely-related sister would provide an ideal model system, since the radiation sensitive and resistant cells would share a common genetic heritage.

In light of this, one purpose of this study was to establish closely-related radioresistant and radiosensitive cell clones to examine the possible genetic basis of radiation resistance or sensitivity. The HCT116 colorectal cell line was chosen because it is a relatively well characterized cell line and also because S phase agents

(in particular, 5-FU) are known to be useful for combining with X-radiation for the treatment of human colorectal cancer.

Several clones of varying radiosensitivity were isolated from the untreated parental cell line. Radiosensitivities of cloned populations of cells by increasing doses of ionizing radiation were analyzed by clonogenic assay. Several clones were isolated and characterized for possible genes involved in radioresistance and/or radiosensitivity.

The objectives of this research was to:

1) identify the mechanisms of cellular death of a radioresistant human melanoma cell line (Sk-Mel-3) and a non-transformed fibroblast cell line (AG1522) following treatment to the type-1 DNA topoisomerase inhibitor (Camptothecin - CPT) and X-radiation.

2) determine the effectiveness of CPT to potentiate fractionated doses of X-radiation in the human tumour colorectal cell line (HCT116), a tumour type already known to respond well to CPT alone treatment, in comparison with the non-transformed fibroblast cell line (AG1522), *in vitro*.

3) determine if selection of radioresistant cells within the human tumour colorectal cell line (HCT116) occurs due to intrinsic radioresistance or due to adaption to the fractionated X-radiation treatment.

4) isolate and characterize human colorectal tumour cell line (HCT116) clones of varying radiosensitivities and determine their cross sensitivities to drug and UV.

5) identify possible genes involved in manifesting the radiation response in these clones, and to confirm the microarray results with quantitative PCR (Q-PCR).

Investigating and understanding the mechanisms involved in the radioresponse is fundamental to understanding the role of radioresistance in radiotherapy. In addition, comprehension of these mechanisms may permit future therapies where the emergence of radioresistance is limited, during treatment and may be important to the advancement of radiotherapy.

1.8 THESIS OUTLINE

This thesis is comprised of six chapters and one appendix. Each chapter is a comprehensive study which addresses a specific objective of the thesis.

Chapter 2 addresses objective one and examines if either type of cell death (necrotic or apoptotic) was more associated with the combination of CPT and X-radiation in a radioresistant melanoma cell line and a normal human fibroblast cell line. The preface to Chapter 3 addresses the second objective of whether camptothecin combined with clinically relevant fractionated doses of X-radiation could potentiate the killing of human colorectal tumour (HCT116), a more sensitive target of CPT and its analogs, but not normal human fibroblast (AG1522) cells. This brief study also lead to the discovery of the effects of fractionated X-radiation treatment alone on HCT116 cells that directed the progress of the remaining chapters. Chapter 3 examines the third objective of determining whether radioresistance occurs because of either an adaptive response to, or by the selection of radiation-resistant cells, from a human colorectal tumour (HCT116) cell line, by the previous fractionated radiation exposure. Objective four is investigated in chapter 4 which was to isolate clones from a nonirradiated parental HCT116 cell line cell line with varying radioresponses and determine if a correlation exists with cell clone radiosensitivity and response to either drug and UV treatment. Chapter 5 addresses the fifth objective of understanding the underlying genetic basis of radiosensitivity. Chapter 6 presents complementary studies which relate to the studies in the previous chapters and enhance the significance of the findings.

Due to the extensive scope of this thesis, each chapter deals with a specific area of study with regard to radiosensitivity, and is therefore comprised of the sections: introduction, materials and methods, results, and discussion. While the findings of each chapter are significant in their contribution to an aspect of radiosensitivity, together they represent a comprehensive examination of the factors which govern cellular radioresponsiveness as well as address the ability of exogenous factors (i.e. chemo-drugs) to modulate sensitivity. This is also discussed in chapter 6 as possible areas for future research.

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2. ABSTRACT

Purpose: We evaluated apoptotic, necrotic and clonogenic cell deaths and inhibition of cell growth in a human melanoma (Sk-Mel-3) and a normal human fibroblast cell line (AG1522) following treatment with camptothecin (CPT) or with concurrent CPT and X-radiation.

Materials and Methods: Apoptotic and necrotic cell deaths were determined morphologically by dual-staining (propidium iodide, acridine orange). Inhibition of cell growth was determined from the number of cells remaining in the culture dish following treatment.

Results: We found, in Sk-Mel-3 cells, that: (a) after CPT alone treatment, both apoptotic and necrotic cell deaths increased significantly ($p<0.05$) relative to untreated controls, (b) after concurrent CPT and radiation treatment, however, only the increase in necrotic cell deaths was significant ($p<0.05$) when compared to cells receiving radiation alone, and (c) all assays of cellular effects/cytotoxicities were consistent in showing that CPT, given alone or with radiation, led to a substantial increase in cell kill. In contrast, for AG1522: (a) there were no significant increases in apoptotic or necrotic cell deaths following either CPT alone or concurrent CPT and radiation, and (b) the clonogenic assay measured

substantially higher cytotoxicities than the other assays.

Conclusion: Necrotic cell deaths were relatively more important than apoptotic cell deaths during concurrent CPT and radiation treatments in Sk-Mel-3, but not AG1522, cells.

2.1 INTRODUCTION

There are several pertinent issues to consider when treating human melanoma. First, melanomas have a wide range of resistance to radiation. Second, melanomas have a propensity to metastasize quickly. Third, metastatic melanoma (MM) is characteristically unresponsive to conventional chemotherapy [32]. Recently, numerous clinical trials have demonstrated that chemoradiation treatments (in particular, concurrent chemotherapeutic and radiation treatments) given to various tumour sites are associated with significantly increased patient survival relative to treatment with either drug or radiation alone [9,25,31]. Chemoradiation treatments have two principal objectives: (a) enhancement of local tumour control through the interaction of the two cytotoxic modalities, and (b) reduction of treatment failure caused by distant metastases through the action of the drug on sites that are not irradiated. Thus chemoradiation treatments can potentially enhance the response of non-resectable primary melanomas to treatment and reduce the incidence of metastases resulting from local therapeutic failure. Chemoradiation may also have a role in the treatment of various MM (dermal, subcutaneous, lymph node, brain).

It is unknown why some melanomas are very radiation-resistant. The melanin content of the cells may be important, however, this issue is controversial [16,36]. It is also unknown why MM is refractory to chemotherapeutic agents. However, there is recent evidence that melanoma cells may be resistant because of a reduced ability to undergo apoptosis following treatment [19,20,32]. It is well established that two important forms of cell death, apoptosis and necrosis, occur in response to treatment with drugs and radiation [4,10,11]. Apoptosis is characterized by distinct cellular changes such as chromatin condensation, membrane

blebbing, cell shrinkage, and, frequently, formation of DNA ladders [4]. Apoptosis typically occurs at protracted times (can be up to several days later) following the inducing event and apoptotic cells are frequently found in the floating fraction of cells growing in culture following treatment with drugs and radiation [10]. Conversely, necrotic cell death is associated with an absence of the cellular characteristics described for apoptosis (i.e. no chromatin condensation, detection of DNA smearing rather than laddering, cell swells rather than shrinks, cell dies soon after necrosis-inducing event) [4].

We, and others [5,8,26,38], have previously shown that camptothecin (CPT) is a useful adjunct to radiation. CPT is a compound derived from the oriental plant Camptotheca acuminata (tree of joy) and is a specific inhibitor of DNA topoisomerase (topo) I. CPT analogs are already being used for cancer therapy. We have reported that CPT and radiation interacted synergistically in plateau-phase cultures (which model non-actively proliferating cells) of radioresistant human melanoma cells [27]. Conversely, CPT and radiation interacted additively in exponential-phase cultures (which model actively proliferating cells) of these cells [30]. In this study, we have evaluated the relevance of the different forms of cell death in these radioresistant human melanoma cells following treatment with CPT alone or with concurrent CPT and radiation. This was done to test the hypothesis that apoptotic and necrotic cell deaths were important following combined treatment with CPT and radiation.

Additionally, we have extended our studies to a normal human fibroblast cell line. Although the normal counterpart of melanoma cells is the melanocyte, there is little evidence that its response is germane to late radiation effects. Late effects of radiation (fibrosis, tissue necrosis), which often limit radiation dosage, are correlated with the response of fibroblasts [6,7,15]. Thus the response of this cell type to combined CPT and radiation is potentially

relevant from a clinical perspective.

2.2 MATERIALS AND METHODS

2.2.1 ***Cell lines and culture:*** Sk-Mel-3 is a very radioresistant, human melanoma cell line originally established from a lymph node metastasis (ATCC) [27]. AG1522 is a human skin fibroblast cell line that is non-tumourigenic, is contact-inhibited and has been previously described [28]. Cells were cultured in a 1:1 mixture of Dulbecco's modified essential medium: F12 Nutrient Mixture (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco LifeTechnologies, Mississauga, ON, Canada), 0.1 mM non-essential amino acids (Sigma), 20 mM Hepes (Boehringer Mannheim) and 10 mM $NaHCO_3$ (Sigma) in a humidified atmosphere of 98% air and 2% CO_2 at 37°C. Cultures in the exponential phase of growth were obtained by seeding 2 X 10^5 viable cells in 25-cm^2 flasks on day 0. Cells were used for experiments on day 3.

2.2.2 ***Drug exposures and irradiation:*** CPT (Sigma) was dissolved in DMSO and appropriate drug concentrations were added to the culture medium covering the cells as previously described [27]. All CPT exposures were for 1 h at 37°C. For concurrent CPT and X-radiation treatments, CPT was added and cells were irradiated at room temperature on a 250 kVp X-ray system (Pantak, CT) at a dose rate of 150 cGy/min. Flasks were immediately returned to the 37°C incubator for the remainder of the hour-long CPT exposure.

2.2.3 ***Colony-forming assay:*** Immediately following treatment with CPT or CPT and radiation, cells were rinsed twice with isotonic citrate saline, trypsinized (0.2 % trypsin/2.5 mM EDTA for 5 min at 37 °C) and counted with an electronic particle counter. Cell suspensions were plated to yield ~50 colonies/60 mm dish after 14 days in a humidified atmosphere of 98 % air, 2 % CO_2. Dishes were stained with methylene blue and colonies containing > 50 cells were scored to assess relative colony formation. Radiation survival curves were fitted with the linear quadratic model $S=\exp(-\alpha D-\beta D^2)$. Both radiation and CPT curves were fitted using Sigma Plot software (SPSS Inc).

2.2.4 ***Inhibition of cell growth/attachment assay:*** Cells were treated with DMSO (i.e. control), 2μM CPT, or 25 μM CPT. The medium was changed at the end of the exposure. Cells were returned to the incubator and kept at 37°C in 98 % air, 2 % CO_2 for various times up to 96 h. At the appropriate times, media was collected and floating cells counted on an Elzone 80 cell counter (Particle Data Inc., Elmhurst, IL). Attached cells were trypsinized and counted following a wash with phosphate buffered saline (PBS).

2.2.5 ***Determination of apoptotic, necrotic and live cells:*** For CPT alone treatment, cells were treated with DMSO (i.e. control), 2 μM CPT, or 25 μM CPT. At the end of the exposure, the medium was changed (after rinsing with non-drug/DMSO containing medium 2X). The cells were incubated at 37°C in 98 % air, 2 % CO_2 for various times up to 120 h. For concurrent CPT and X-radiation treatments, cells were irradiated in the presence of CPT (as described above) and returned to the incubator for 48 h. At this time, the cells were rinsed

with PBS and trypsinized (0.2 % w/v, 2.5 mM EDTA, 5 mins., 37°C). Both detached and attached cells were pooled for determination of apoptotic, necrotic and live cells.

Relative percentages of apoptotic, necrotic and live cells were determined using a well established assay [35]. Briefly, cells were simultaneously stained with 5 μg/ml propidium iodide (PI) and 50 μg/ml acridine orange (AO) in PBS. The morphologies of the cell nuclei were viewed under a fluorescent microscope. Apoptotic and live cells excluded PI because of their intact membranes and were stained green by AO. However, apoptotic cells were clearly distinguishable by their condensed, or fragmented, chromatin and cell membrane blebbing. Conversely, necrotic cells were stained red by PI because of the compromised integrity of their cellular membranes. At least 200 cells were counted in each field.

2.2.6 ***Statistics:*** All experiments were repeated at least three times. Within each experiment, either duplicate or triplicate plates were scored for each dose for the clonogenic assays. Results are presented as mean ± standard error of mean (SEM). For the determination of increase in apoptotic or necrotic cells following treatment, comparisons were made against the untreated (for CPT alone treatments) or irradiated alone (for concurrent CPT and X-radiation treatments) controls using the one-tailed Student's t-test. For all statistical tests, a $p < 0.05$ value was considered significant.

2.3 RESULTS

2.3.1 ***Relative colony formation:*** Sk-Mel-3 cells were significantly more resistant to CPT

than AG1522 cells (Fig. 1). Although the difference in relative colony formation (RCF) was small at a dose of 2 μM CPT, the difference was ~3X at a dose of 25 μM CPT. The presence of sensitive subpopulations in these two cell lines was clearly shown by the shapes of the curves. The sensitive subpopulation, which comprised ~40 % of Sk-Mel-3 cells, agrees well with our previous finding that S-phase cells form ~35 % of exponential-phase cultures of this cell line [29]. The sensitive subpopulation of ~80 % of AG1522 cells, however, is much larger than the S-phase subpopulation which comprise ~33 % (unpublished data) of this cell line.

Fig. 2 shows the RCF levels following radiation alone and concurrent CPT and X-radiation treatments. The CPT dose (25 μM) was chosen to be in the plateau regions of the survival curves of both cell lines (see Fig. 1). The addition of CPT to X-radiation significantly increased the killing of both cell lines without substantially changing the shapes of the radiation survival curves. This confirmed our previous findings that the interaction between CPT and radiation was essentially additive in exponential cultures of these cells. Sk-Mel-3 cells were again more resistant than AG1522 cells to both X-radiation alone or concurrent CPT and X-radiation. At a RCF level of 0.1, this difference was ~1.8X for X-radiation alone and ~2.5X for CPT and X-radiation.

2.3.2 ***Inhibition of cell growth/attachment assay:*** There was a significant decrease in the number of Sk-Mel-3 cells that remained attached to the culture vessel after treatment with CPT and subsequent incubation of the treated cells at 37°C for up to 96 h in the absence of CPT (Fig. 3a). More cells were lost or released into the media following a high (25 μM, chosen to be in the plateau region of the survival curve of Fig. 1) than a low (2 μM, chosen

Figure 1. Relative colony formation curves for Sk-Mel3 and AG1522 cell lines following treatment with CPT (1 h, 37°C).

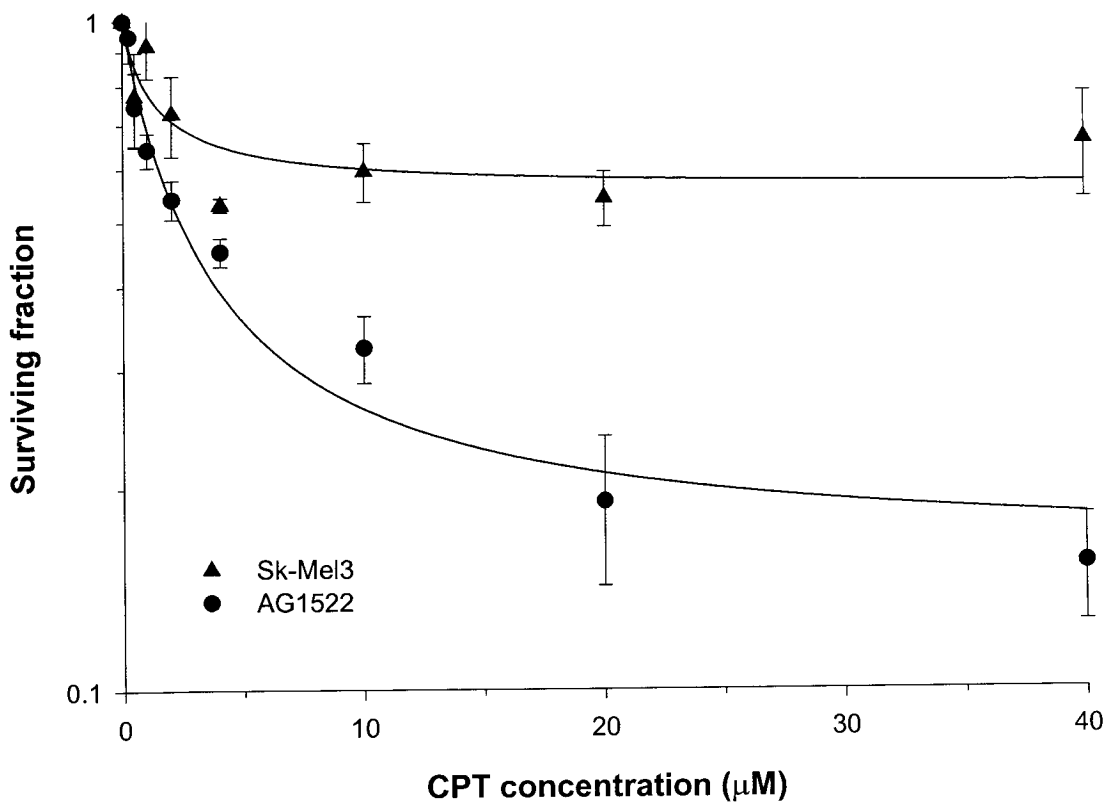
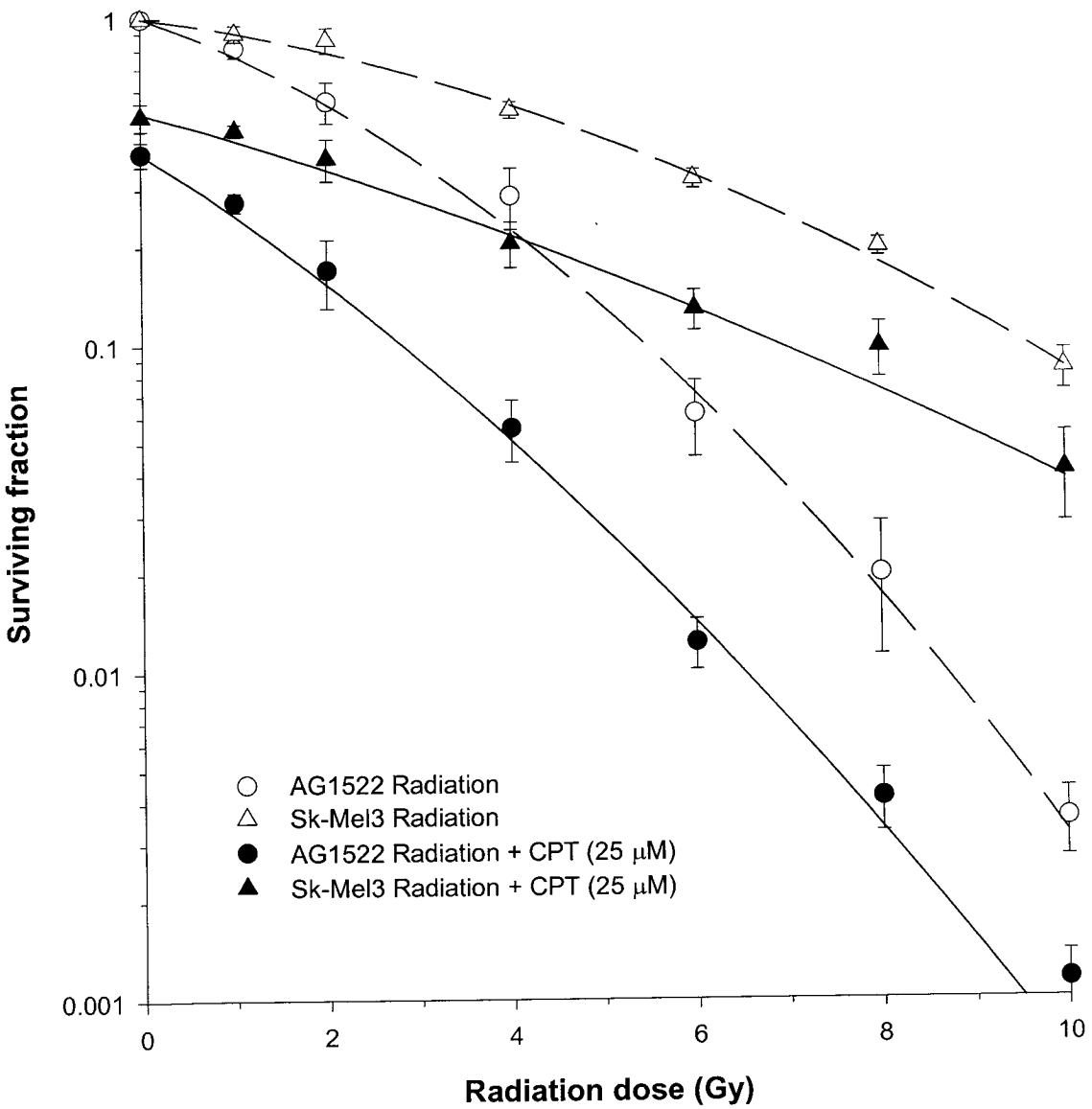

1
0.1
Surviving fraction
0
10
20
30
40
CPT concentration (μM)
Sk-Mel3
AG1522

Figure 2. Relative colony formation curves for Sk-Mel-3 and AG1522 following radiation alone or concurrent CPT and radiation. CPT exposure was 1h at 37 °C.


1
0.1
0.01
0.001
Surviving fraction
0
2
4
6
8
10
Radiation dose (Gy)
AG1522 Radiation
Sk-Mel3 Radiation
AG1522 Radiation + CPT (25 μM)
Sk-Mel3 Radiation + CPT (25 μM)

to be in the initial part of the survival curve of Fig. 1) dose of CPT. Conversely, most AG1522 cells remained attached to the culture vessel under the same conditions of treatment and post-treatment incubation and this was true for both concentrations (2, 25 μM) of CPT (Fig. 3b).

To determine whether the treatments were cytotoxic or cytostatic, we expressed the number of attached cells at various times following treatment with CPT on the basis of the initial number of attached cells (normalized to 100 %). Relative to the untreated control, 2 μM CPT prevented an increase in the number of Sk-Mel-3 cells whereas 25 μM CPT actually caused a substantial reduction to fewer than the initial number of cells by 96 h post-treatment (Fig. 4a). However, both these doses were actually cytotoxic, not cytostatic, since they caused a detachment of cells into the media especially at times > 48 h (Fig. 3a). In contrast, both concentrations of CPT clearly prevented an increase in cell numbers in AG1522 (Fig. 4b) and were mainly cytostatic, not cytotoxic, since few AG1522 cells were released into the media under these treatment conditions (Fig. 3b).

2.3.3 ***Apoptotic versus necrotic cell deaths:*** Because of the obvious differences in detachment and release of dead or dying cells into the surrounding media after treatment manifested by these two cell lines, we evaluated the total number of cells (i.e. attached plus floating in medium) in subsequent experiments. Fig. 5 shows the time course of CPT-induced cell deaths. Relative to untreated controls, there was a significant increase ($p<0.05$) in both apoptotic and necrotic cell deaths after 25 μM, but not 2 μM, CPT in Sk-Mel-3 (Fig. 5a). For the latter (2 μM CPT), only the increase in necrotic death at 96 h was significant. Conversely, there were no significant increases in either forms of cell death in AG1522 after

Figure 3a, b. The number of cells (**a** Sk-Mel-3, **b** AG1522) that remain attached following treatment with CPT (1 h, 37°C) and post-treatment incubation at 37°C for various times up to 96 h. The numbers of attached cells are expressed as a percentage of the total (i.e. attached plus floating in media) number of cells for both cell lines.

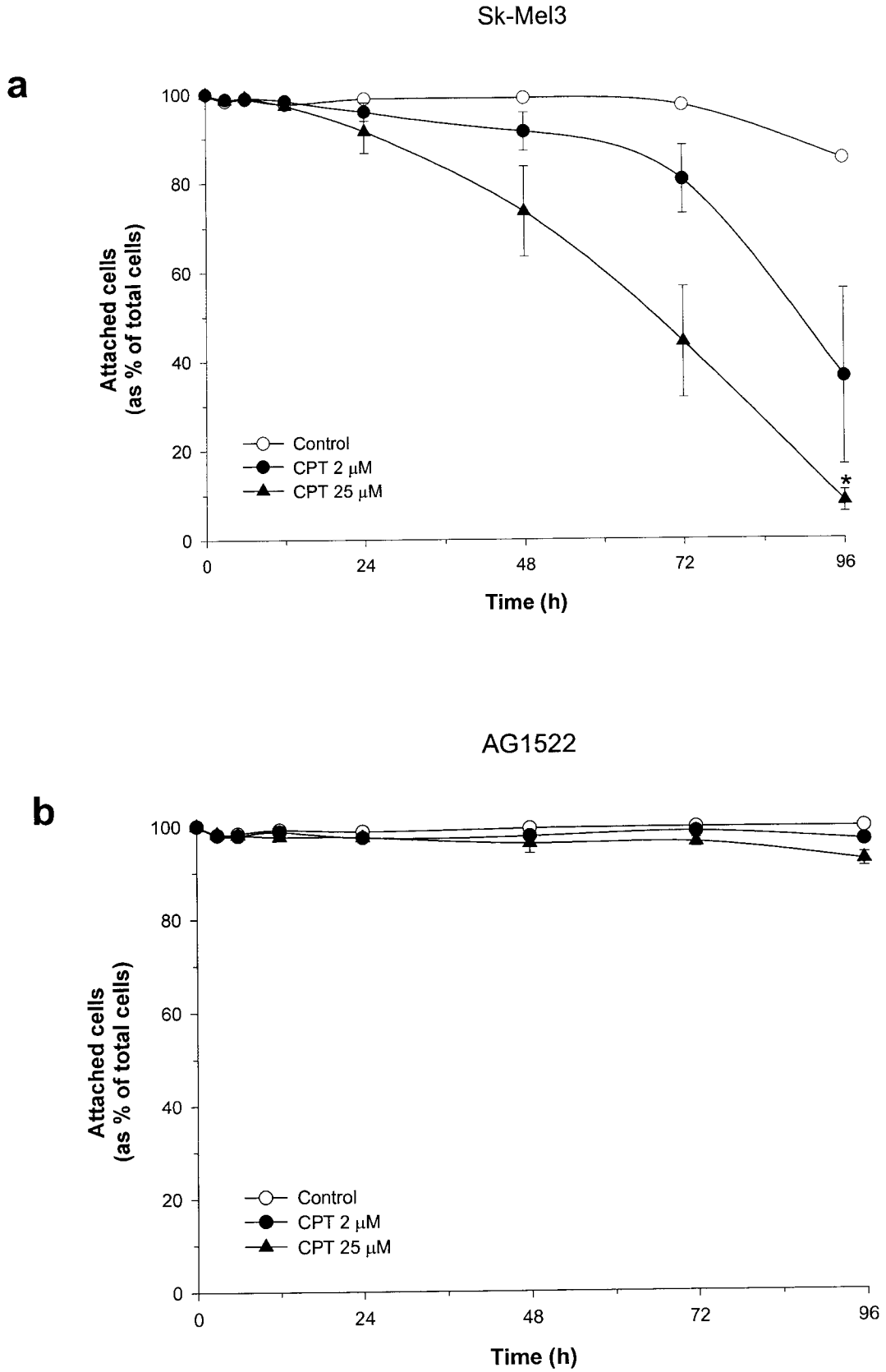

Sk-Mel3
a
100
80
60
40
20
0
Attached cells
(as % of total cells)
Control
CPT 2 μM
CPT 25 μM
*
0
24
48
72
96
Time (h)
AG1522
b
100
80
60
40
20
0
Attached cells
(as % of total cells)
Control
CPT 2 μM
CPT 25 μM
0
24
48
72
96
Time (h)

Figure 4a, b. Effect of treatment with CPT (1 h, 37°C) and post-treatment incubation at 37°C for various times up to 96 h on the growth of the cells (**a** Sk-Mel-3, **b** AG1522).

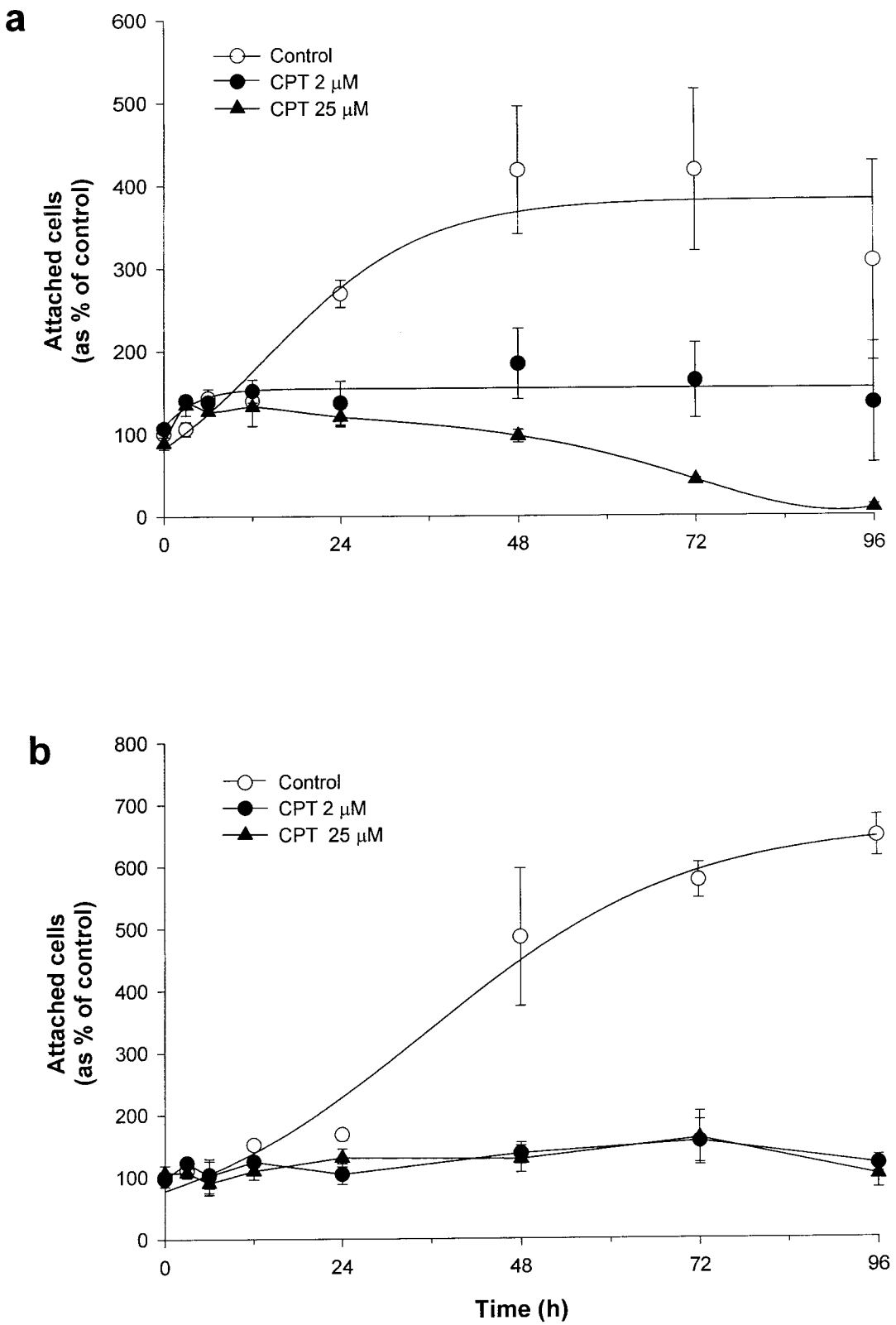
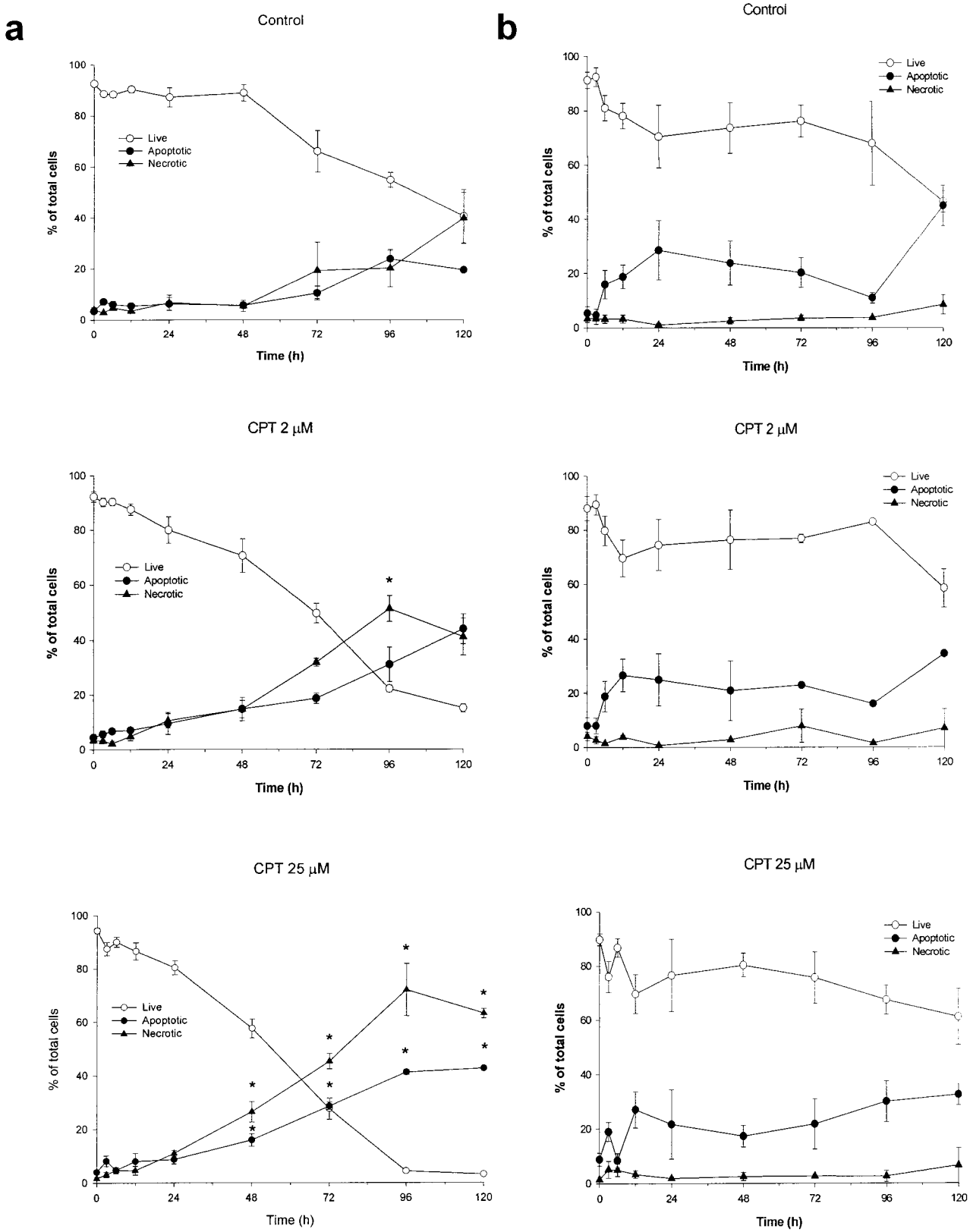

a
600
500
400
300
200
100
0
Attached cells
(as % of control)
Control
CPT 2 μM
CPT 25 μM
0
24
48
72
96
b
800
700
600
500
400
300
200
100
0
Attached cells
(as % of control)
Control
CPT 2 μM
CPT 25 μM
0
24
48
72
96
Time (h)

Figure 5a, b. The relative number of apoptotic, necrotic and live cells (**a** Sk-Mel-3, **b** AG1522) following treatment with CPT (1 h, 37°C) and post-treatment incubation for various times up to 120 h. Attached cells were pooled together with floating cells in these experiments. *Top panels* control cells treated only with DMSO (*control*), *center panels* 2 μM CPT, *bottom panels* 25 μM CPT. *P<0.05 vs control

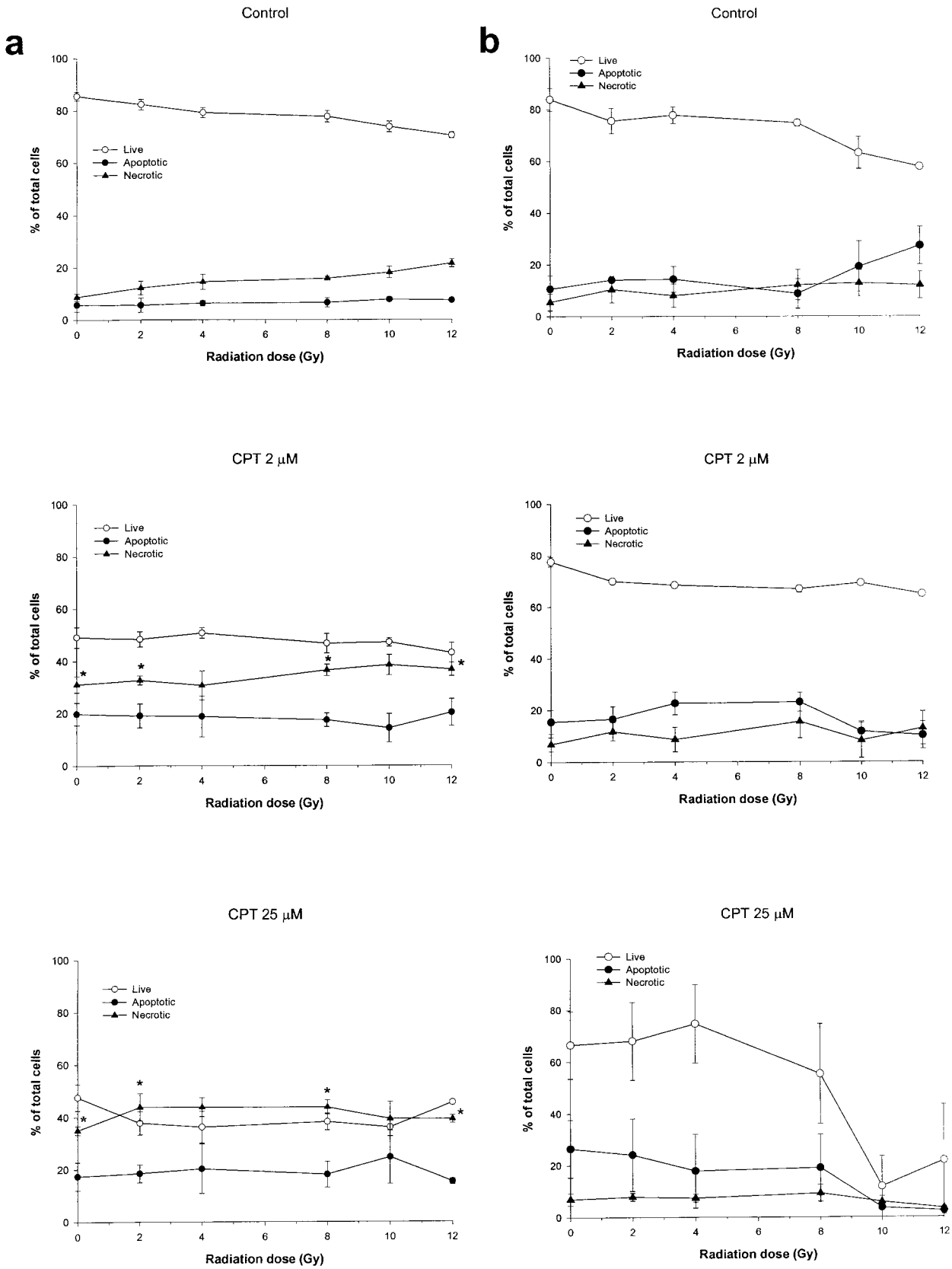

a
b
Control
Control
CPT 2 μM
CPT 2 μM
CPT 25 μM
CPT 25 μM
Live
Apoptotic
Necrotic
% of total cells
Time (h)
0
20
40
60
80
100
0
24
48
72
96
120
*

treatment with CPT alone (Fig. 5b).

After concurrent CPT and X-radiation treatments in Sk-Mel-3 cells, there were again increases in the number of apoptotic and necrotic cell deaths relative to the cells receiving irradiation alone (Fig. 6a). However, only the increases in necrotic cell deaths were significant ($p<0.05$) and this was true for both 2 and 25 μM CPT given with X-radiation. For AG1522, there were small increases in apoptotic cell deaths following both 2 and 25 μM CPT with X-radiation, however, these increases were statistically insignificant (Fig. 6b). Thus there were no significant increases in either forms of cell death for both concentrations of CPT given concurrently with radiation in AG1522.

Necrotic cell deaths were relatively more frequent than apoptotic cell deaths in Sk-Mel-3 after both CPT and concurrent CPT and X-radiation (Fig. 5a, 6a) whereas the reverse was true in AG1522 (Fig. 5b, 6b).

Figure 6a, b. The relative number of apoptotic, necrotic and live cells (**a** Sk-Mel-3, **b** AG1522) as a function of radiation dose assayed at 48 h post-treatment with concurrent CPT and X-radiation. CPT exposure was for 1h at 37°C. Attached cells were pooled with floating cells in these experiments. *Top panels* control cells treated with irradiation and DMSO, *center panels* 2 μM CPT with radiation, *bottom panels* 25 μM CPT with radiation. *P<0.05 vs irradiated-alone control


a
Control
% of total cells
Radiation dose (Gy)
Live
Apoptotic
Necrotic
b
Control
CPT 2 μM
CPT 25 μM
0
20
40
60
80
100
0
2
4
6
8
10
12
*

2.4 DISCUSSION

The incidence of human melanoma is increasing at a rate that exceeds that of all other solid tumours [2]. Although surgery is usually curative when melanomas are detected at an early stage, the outlook for melanoma that has metastasized beyond the regional lymph nodes remains bleak (median survival is in the order of months). Further, metastases are challenging to treat because they frequently involve multiple sites (including to skin, lung, liver, bone, brain) [2]. Radiation is being re-evaluated as a treatment modality in primary locoregional management of melanomas because some melanomas do respond to radiation clinically [1]. Chemoradiation, acting either through a synergistic or additive interaction between the drug and radiation, may therefore be potentially useful for decreasing the incidence, and/or for the treatment, of MM.

There is strong evidence for the genetic basis of apoptosis with some genes being regarded as pro-apoptotic (e.g. wt p53, bax) [21,22,24,37] and others as anti-apoptotic (p21/waf1/cip1, bcl-2) [13,14]. More specifically, adenovirus-driven ectopic expression of p21/waf1/cip1 substantially protected against p53-dependent apoptosis in human melanoma cells [13]. Recently, it has been claimed that some proto-oncogenes (Bcl-2/Bcl-xL) and ICE-like proteases may modulate both apoptotic and necrotic cell deaths, indicating that apoptosis and necrosis may share some common pathways and/or mediators [12,33]. This further suggests that the relationship between apoptotic and necrotic deaths may be important in manifesting cytototoxic stresses due to chemoradiation treatments. Modulation of apoptotic

and necrotic cell deaths on a genetic basis may ultimately permit enhanced killing of melanoma cells and decreased cytotoxicity to normal cells, thereby leading to an increase in clinical therapeutic index. Our intention in this study therefore was to examine the relevance of both these forms of cell death following combined CPT and radiation treatment. Note that: (a) although recent studies have documented the importance of the apoptotic form of death to melanoma cells treated with CPT [19,20], there is very little data in regard to the relative importance of apoptotic to necrotic cell deaths in human melanoma and human normal fibroblast cells treated with combined CPT and radiation, and (b) the concentrations of CPT used in this study are higher than typical clinical exposures.

The main findings of this study were: (a) in Sk-Mel-3, both necrotic and apoptotic cell deaths were important following treatment with CPT alone, however, necrotic death was relatively more important following CPT combined with X-radiation, (b) in AG1522, both apoptotic and necrotic cell deaths were insignificant after CPT alone or combined with X-radiation, and (c) in Sk-Mel-3, all four methods of determining treatment-related cellular effects (apoptotic, necrotic, clonogenic cell deaths, inhibition of cell growth) were consistent insofar as demonstrating that CPT given alone, or with X-radiation, led to a substantial increase in cytotoxicity, however, this was not the case with AG1522. Thus our present studies have provided evidence that necrotic cell death may be relatively more important than apoptotic cell death during combined CPT and radiation treatments in Sk-Mel-3, but not AG1522, cells.

The apparent discrepancy in the AG1522 results merits further consideration. In this cell line, three assays (necrotic and apoptotic cell deaths, inhibition of cell growth) were consistent insofar as showing that these treatments (2, 25 μM CPT) had minimal toxicity to

these cells. Curiously, the clonogenic surviving fraction assay showed that 25 μM CPT in particular had killed a substantial proportion (~ 80 %) of the AG1522 cells. Similarly, the clonogenic assay also detected substantially higher cytotoxicity following 25 μM CPT and radiation as compared to the apoptotic/necrotic assays. It should be noted, however, that the clonogenic assay detected the loss of clonogenic potential from all possible causes, including apoptosis and necrosis, occurring over a longer period (14 days) after treatment than the apoptotic/necrotic and growth inhibition assays (120 h for CPT alone treatment or 48 h for CPT combined with radiation). This, therefore, suggested that either the AG1522 cells were dying in the period after the completion of the apoptotic/necrotic assays or that the additional steps of trypsinizing and replating the cells for the clonogenic assay might be related to the apparent discrepancy in the perceived killing of these cells. Future studies are required to determine which is the case. Interestingly, while CPT and its analogs can induce p53-dependent apoptosis in human ovarian carcinoma cell lines, these drugs can also induce p53-independent cell death as measured by the clonogenic assay [23]. Whether this was also happening in the AG1522 cells (i.e. p53-independent cell death being measured by the clonogenic assay) is presently unknown.

One factor that might potentially confound the evaluation of the relative importance of apoptotic to necrotic deaths in our present study is the possibility of apoptotic cells undergoing a subsequent secondary necrosis in the absence of phagocytosis occurring in vitro (note: even in the presence of phagocytosis, there might be an increase in necrotic cells if the rate constant for the conversion of apoptotic to necrotic cells is larger than that for the formation of apoptotic cells). Secondary necrosis might artificially increase the proportion of necrotic relative to apoptotic cells that was detected by the apoptotic/necrotic assay.

However, secondary necrosis was unlikely to be important in AG1522 under our experimental conditions. This is because both apoptotic and necrotic deaths were very low in this cell line following both types of treatment (CPT± radiation). While it is possible that secondary necrosis might have some contribution at later time points (>72 h after CPT alone) in Sk-Mel-3, it is also unlikely to be important at the earlier time points. Specifically, whereas apoptosis was still relatively low, necrosis was already significantly increased at both 48 and 72 h post treatment with 25 μM CPT (Fig. 5a). In particular, the actual increase in apoptotic cells relative to untreated controls was very small and statistically insignificant (~8%) for the 48 h point after 25 μM CPT; in contrast, the relative increase in necrotic cells was larger (~20%) and statistically significant (Fig. 5a). Similarly, at 48 h following combined CPT and radiation treatments, there were already significant increases in necrotic deaths in Sk-Mel-3 occurring in the absence of significant increases in apoptotic deaths (Fig. 6a). Hence secondary necrosis was also unlikely to be important after combined CPT and X-radiation in Sk-Mel-3 under our experimental conditions.

Various cellular mechanisms have been proposed as potentially pertinent to the expression of resistance to chemotherapeutic agents by melanoma cells. These mechanisms include alterations in the expressions of the multidrug resistance associated protein (MRP), glutathione and related enzymes, the target enzymes (i.e. DNA topo I and II) of the DNA topo-targeting drugs, and various genes (N-ras, bax/bcl-2, p53, Apaf-1) involved in the expression of cell death [3,32] [34]. There has been particular recent interest in elucidating the roles of p53 in human melanoma because: (a) p53 is apparently commonly overexpressed in MM but not in primary melanoma [17,18], and (b) mutations of the p53 gene occur in 25-30% of MM and cultured melanoma cell lines [32]. Thus mutation of the p53 gene may be a

useful indicator for drug resistance in this disease. Consistent with this view, melanoma cells expressing wt p53 were much more sensitive to treatment with CPT relative to those expressing mt p53 [20]. Further, melanoma cells overexpressing mt p53 show reduced sensitivity to CPT and lower (2-3 fold) CPT-induced apoptosis [19]. Note that p53 is upregulated, especially significantly at the later time points (~96 h), after treatment with CPT in AG1522 but not in Sk-Mel-3 cells (data not shown). Further, Sk-Mel-3 cells also have hardly detectable endogenous amounts of p53, suggesting that it has dysfunctional wt p53 whereas AG1522 has functional wt p53 (data not shown). Interestingly, a recent study has proposed that loss of Apaf-1, a cell death effector that acts with cytochrome c and caspase 9, is responsible for abrogating p53-dependent apoptosis in MM cells [34].

In summary, our present study has demonstrated that necrotic cell death may be relatively more important than apoptotic cell death in Sk-Mel-3, but not AG1522, cells being treated with combined CPT and radiation.

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NOTE: Western blots that were not shown in the published report presented in **CHAPTER 2 - COMPARISON OF APOPTOTIC, NECROTIC AND CLONOGENIC CELL DEATHS AND INHIBITION OF CELL GROWTH FOLLOWING CAMPTOTHECIN AND X-RADIATION TREATMENTS IN A HUMAN MELANOMA AND A HUMAN FIBROBLAST CELL LINE** (i.e. data not shown) are displayed in chapter 6.7.0 of the appendix.

PREFACE TO CHAPTER 3

THE TOPOISOMERASE I INHIBITOR, CAMPTOTHECIN, POTENTIATES THE KILLING OF HUMAN COLORECTAL TUMOUR, BUT NOT NORMAL, CELLS TO FRACTIONATED HIGH DOSE RATE X-RADIATION

This unpublished data provides the link between chapters 2 and 3.

2.6 INTRODUCTION

The previous chapter examined the importance of cell death in combinational drug and acute radiation treatment in a radioresistant melanoma as compared to a relatively radiosensitive fibroblast cell line. Similar to this study, the majority of *in vitro* studies apply radiation as a single high dose whereas the clinicial procedures typically deliver the radiation dose in small fractions. A problem with determining survival from single dose radiation treatments *in vitro* is that they fail to correlate with the radiocurability of the tumour *in vivo* (Dahlberg *et al.*, 1999). Moreover, single dose survival curves cannot display the effects of sublethal damage repair, shifts in cell cycle, and repopulation of the tumour by radioresistance cells which has been known to occur following fractionated irradiation *in vivo* (Aruga *et al.*, 1995; Dahlberg *et al.*, 1999; Trott, 1999).

Intriguingly, there are very few studies that examined the interaction between CPT analogs and fractionated radiation (Boscia *et al.*, 1993; Kirichenko and Rich, 1999; Lamond *et al.*, 1996). For example, the effects of γ-irradiation combined with 9-aminocamptothecin (9-AC) on a mouse mammary cancer and the gastrointestinal tract were compared for single (15 Gy) and fractionated (2 Gy * 14 consecutive days) treatment. It was found that a single dose of 9-AC administered with single fraction irradiation was ineffective, however, when 9-AC was combined with fraction radiation a significant delay in tumour regrowth was observed with a maximum

sensitizer enhancement ratio (SER) of 1.7 at 10% survival, depending on the dose of 9-AC (Kirichenko and Rich, 1999). Similarly, in another murine mammary carcinoma, the CPT analog, topotecan (TPT) and fractionated radiation gave a higher SER after only five fractions (3 Gy per day) relative to a single radiation dose (Teicher *et al.*, 1993). Also, topotecan given between two fractions of radiation enhanced lethality in a radioresistant human melanoma (U1-Mel) cell line giving an SER of 1.7 (Lamond *et al.*, 1996).

Despite the antitumour activity potential of topo poisons against human melanoma (Ng *et al.*, 1994), several phase II clinical trials showed the drug to be inactive in patients with recurrent and/or metastatic malignant melanoma (Ellerhorst *et al.*, 2002; Kraut *et al.*, 1997). The tumour sites that respond to treatment with topo poisons are colorectal, ovarian, small-lung cancer and pancreatic and are therefore good candidates for combined treatment with fractionated radiation (Pizzolato and Saltz, 2003; Rothenberg *et al.*, 1996; Takigawa *et al.*, 2003; Takimoto and Arbuck, 1997).

For this reason, we have chosen to determine the effectiveness of CPT to potentiate fractionated (2 Gy fractions over 10 consecutive days) doses of X-radiation in the human tumour colorectal cell line (HCT116), a tumour type already known to respond well to CPT analog alone treatment, *in vitro*. A comparison with the normal fibroblast cell line (AG1522) have been included because normal tissue toxicity determines the maximal allowable IR dose delivered to the tumour tissue. Our

hypothesis was that CPT could significantly enhance the killing of human tumour, but not normal, cells to fractionated, but not single, high dose rate X-radiation with clinically relevant doses of 2 Gy.

2.7 MATERIALS AND METHODS

2.7.1 *Cells and culture conditions:*

Normal human foreskin fibroblast (AG1522) cells and the human colorectal tumour (HCT116) cell line are available from the American Type Culture Collection (ATCC). Cells were cultured at 37°C in DMEM/Ham's F12 1:1 mix containing 10% (v/v) fetal bovine serum and in a humidified atmosphere of 98% air, 2% CO_2. Experiments were performed with exponential cultures by seeding $2x10^5$ cells into a 25 cm^2 flask on day 0 and using these cells for experimentation on day 3. Fractionated X-radiation treated cell cultures were originally plated at the above-mentioned cell density and received fresh media changes every 3 days over the 10-day period. The plated cell numbers used for acute radiation sensitivity determination after fractionated X-radiation treatment were similar to the untreated-acute experiments. Additionally, surviving fractions determined during the fractionated X-radiation were based on the plating efficiency from the untreated cells at day 0.

2.7.2 *Irradiation:*

Cells were irradiated with a 250 kVp X-ray unit (Pantak, CT) at room temperature at a dose-rate of 150 cGy/min. Fractionation was performed as a daily single dose of 2 Gy for 10 consecutive days and immediately returned to the incubator.

2.7.3 *Drug treatment:*

Camptothecin (CPT) at a final concentration of 10 μM was dissolved in DMSO and were applied to cells for the duration of the treatment and refreshed every 3 days. For acute X-radiation exposures, CPT (10 μM) was given 1-hour prior and during irradiation before plating for the clonogenic assay. Cells not given drug, received a DMSO vehicle control in the culture medium.

2.7.4 *Colony forming assay:*

Exponentially growing cells were collected daily and immediately following irradiation, rinsed twice with isotonic citrate saline, trypsinized (0.2% trypsin/2.5mM EDTA for 5 min at 37°C) and counted with an electronic particle counter. Suspensions were plated to yield ~50 colonies/ 60mm dish after 14 days in a humidified atmosphere of 98% air, 5% CO_2. Dishes were stained with methylene blue and colonies containing >50 cells were scored to assess survival. Survival curves were fitted with the linear quadratic model $S=\exp(-\alpha D-\beta D^2)$, using Sigma Plot

software (1986-1997 SPSS Inc) and normalized according to Chapter 6.2.0 of the appendix.

The sensitizer enhancement ratio (SER) was calculated from the resulting normalized dose-response curves that were fitted using a second-degree polynomial regression analysis, yielding a linear quadratic equation. SER is a ratio of dose reduction at an iso-survival fraction caused by a chemical radiosensitizer and when no sensitizer is used. The generated SER's at the 10% and 1% survival levels from the clonogenic survival curves determine what combination is most effective. For example a high SER for the tumour cell line relative to the normal cell line will exhibit a possible therapeutic gain. SER value higher than 1.5 will demonstrate a high efficacy whereas a value less than 1 will show an adverse antagonistic effect between the drug and radiation (Phillips *et al.*, 1986; Revesz and Palcic, 1985; Strunz *et al.*, 2002).

2.8 RESULTS AND DISCUSSION

Figure 1 shows a direct comparison between survival curves of AG1552 and HCT116 cells with single high dose irradiations. HCT116 cells appear to be substantially more resistant to the single radiation doses as compared to the normal fibroblast cells. Interestingly, a fractionation regiment of ionizing radiation (IR) appears to spare the AG1522 cells more than the HCT116 (Figure 2) since HCT116 and AG1552 fractionated survival curves are approximately identical. This result is not surprising since clinical practice has shown that fractionated radiation therapy spares normal tissues and in so doing reduces late radiation toxicity (Baumann *et al.*, 2003; Sasaki and Mashiyama, 1985; Thames, 1984).

The sparing of AG1522 cells by fractionated irradiation may allow the fibroblasts to repair radiation-induced DNA damage more efficiently than the HCT116 cells because of the time given between fractions, typical of a schedule of radiotherapy given in the clinic (Mu *et al.*, 2003). The discrepancy of the radioresponse between acute single dose and fractionated doses is supported by Dahlberg, who demonstrated that the survival response to acute doses of ionizing radiation do not necessarily reflect what is given in the clinic and can not be used to predict radiocurability since radiation is usually given as fractions (Dahlberg *et al.*, 1999).

Figure 1. Survival curves for AG1522 fibroblast cells and HCT116 colorectal cells after exposure to acute doses of ionizing radiation. Data points represent the mean and error bars are represented as the standard error of the mean of three independent experiments.

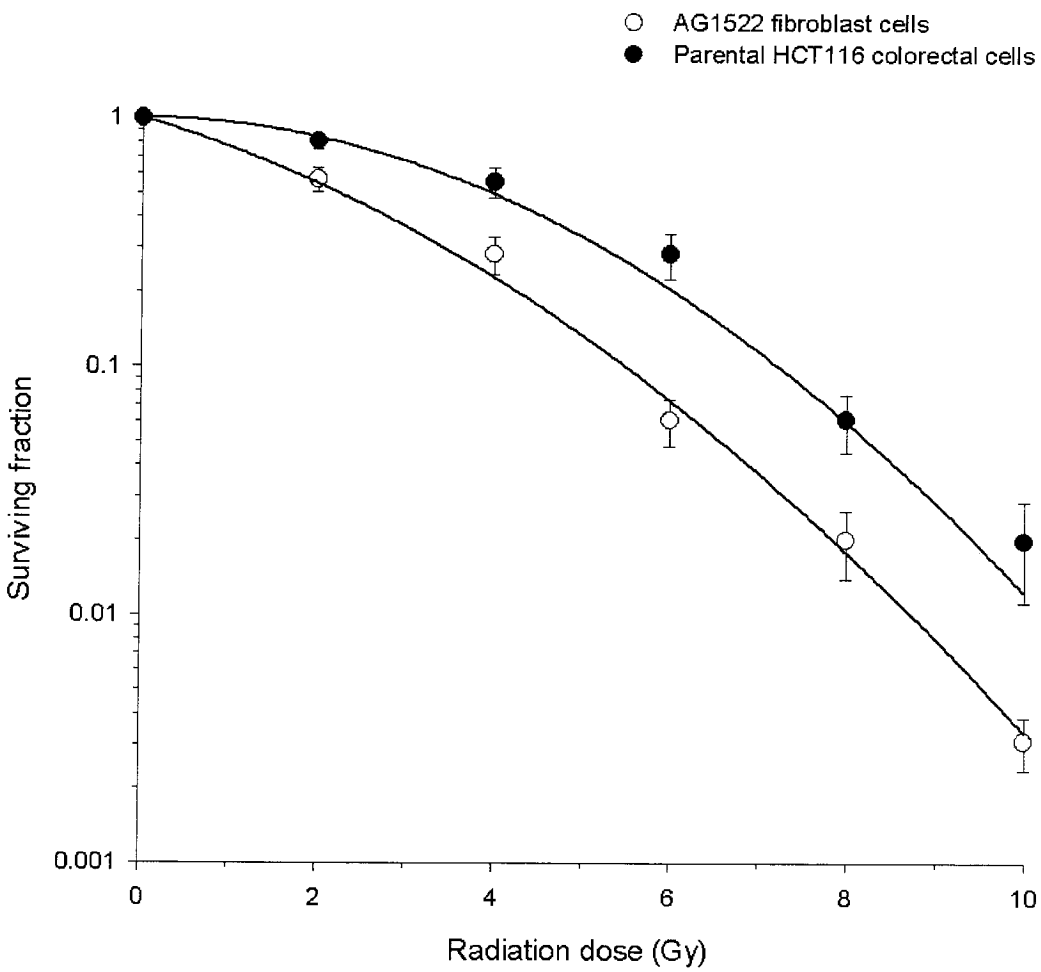
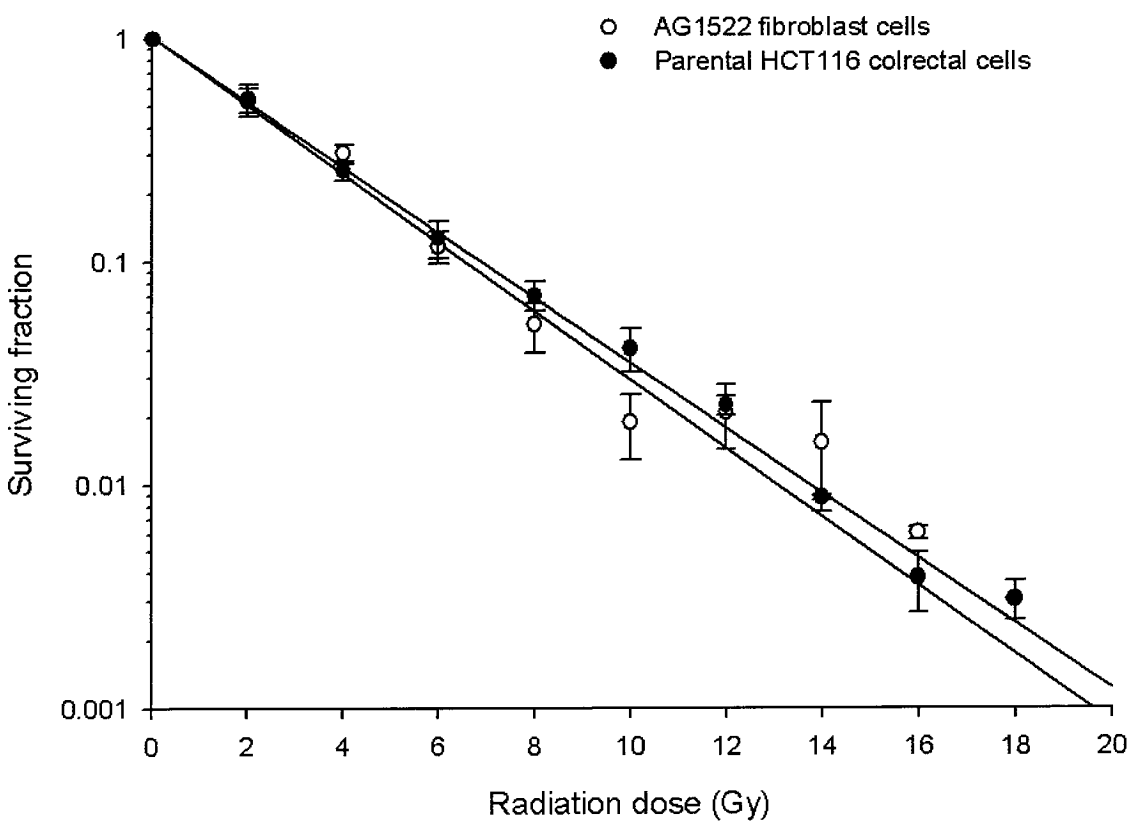

AG1522 fibroblast cells
Parental HCT116 colorectal cells
Surviving fraction
1
0.1
0.01
0.001
0
2
4
6
8
10
Radiation dose (Gy)

Figure 2. Survival curves for AG1522 fibroblast cells and HCT116 colorectal cells after exposure to daily single doses of 2 Gy for 10 fractions of IR. Data points represent the mean and error bars are represented as the standard error of the mean of three independent experiments.


AG1522 fibroblast cells
Parental HCT116 colrectal cells
Surviving fraction
1
0.1
0.01
0.001
0
2
4
6
8
10
12
14
16
18
20
Radiation dose (Gy)

In this case, an additional agent is necessary to enhance killing of the tumour cells while minimizing killing of the fibroblasts cells since the toxicity of either of the two cell lines are similar following fractionated treatment. Fractionated irradiation can select for radioresistant S-phase cells within a tumour (Tennvall *et al.*, 1993), which would allow for specific targeting by the type-1 DNA topoisomerase inhibitor, camptothecin (CPT) or its clinically relevant analogs. Boothman et al. showed in a radioresistant human melanoma cell line that CPT must be present during or immediately after irradiation to have the maximal cytotoxic effect (Boothman *et al.*, 1992). Therefore, CPT (10 μM) was given over the 10 days of fractionated irradiation and refreshed every 3 days. Combined treatment with CPT and acute IR showed additive effects with no evidence for interaction in the cells (figures 3a & 3b). Interestingly, a modest increase in survival was seen with the colorectal cells when acute IR was combined with CPT.

However, treatment with the drug and fractionated IR had significantly reduced clonogenic survival in both cell lines exposed to both CPT and fractionated IR (figures 3a & 3b). Moreover, colorectal cells responded in a highly sensitive manner. No surviving colony was detected after just 2 fractions of IR whereas the AG1522 cells were unable to develop colonies after 3 days in the presence of CPT (figure 3a & 3b).

Figure 3a. A diagram illustrating the survival curves for AG1522 fibroblast cells after exposure to acute and fractionated doses of ionizing radiation with and without concurrent treatment with 10 μM CPT. Data points represent the mean and error bars are represented as the standard error of the mean of three independent experiments. Data points without error bars are represented as the mean of triplicate samples of one experiment.

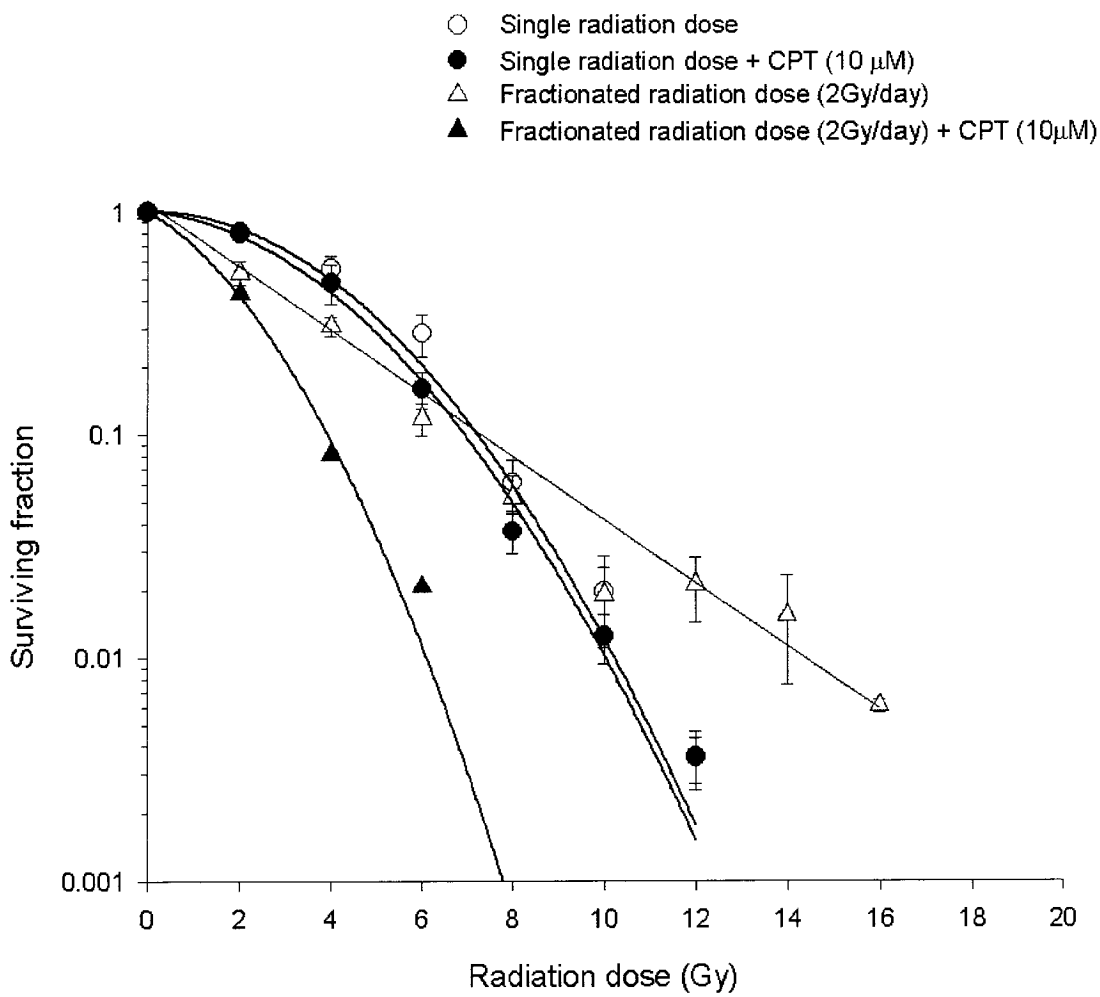
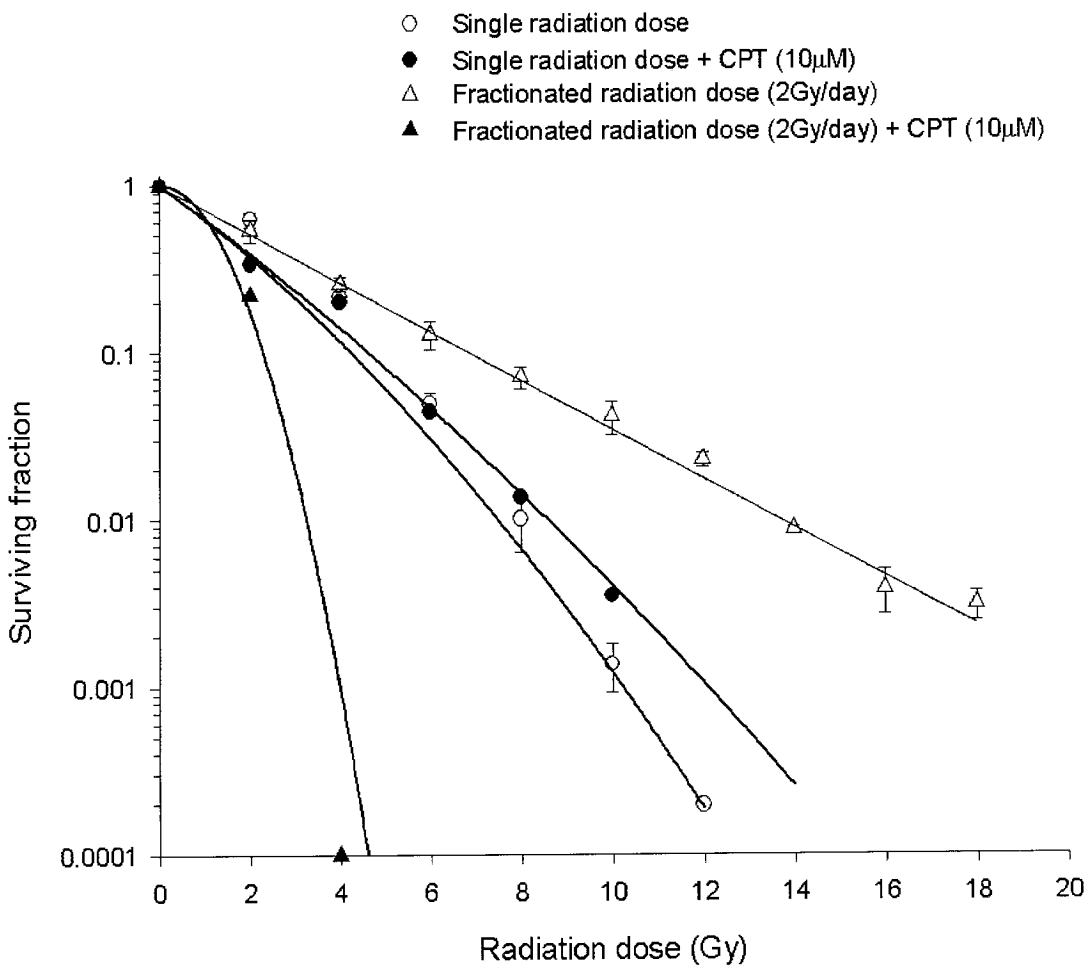

Single radiation dose
Single radiation dose + CPT (10 μM)
Fractionated radiation dose (2Gy/day)
Fractionated radiation dose (2Gy/day) + CPT (10μM)
Surviving fraction
1
0.1
0.01
0.001
0
2
4
6
8
10
12
14
16
18
20
Radiation dose (Gy)

Figure 3b. A diagram illustrating the survival curves for HCT116 colorectal cells after exposure to acute and fractionated doses of ionizing radiation with and without concurrent treatment with 10 μM CPT. Data points represent the mean and error bars are represented as the standard error of the mean of three independent experiments. Data points without error bars are represented as the mean of triplicate samples of one experiment.


Single radiation dose
Single radiation dose + CPT (10μM)
Fractionated radiation dose (2Gy/day)
Fractionated radiation dose (2Gy/day) + CPT (10μM)
Surviving fraction
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
12
14
16
18
20
Radiation dose (Gy)

Tumour cell lines that respond well to CPT or any another sensitizing agent are expected to give a high sensitizer enhancement ratio (SER) (e.g., > 1.5) when combined with IR (Phillips *et al.*, 1986; Revesz and Palcic, 1985; Strunz *et al.*, 2002). We found that the SER at the 10% survival level for fractionated radiation were, respectively, 2.7 vs. 1.7 for these two cell lines. This data clearly suggests that CPT or possibly its analogs are useful adjuncts to fractionated radiation.

Additional experiments of the parental HCT116 cells were then proposed to further characterize the response of these cells to X-radiation and CPT. These cells were to be collected following irradiation with a similar treatment of ten daily 2 Gy fractions and given varying single acute doses of X-radiation in combination with CPT, to determine the effect of fractionated X-radiation has on the acute radiation response of these cells in the presence of CPT. Interestingly, HCT116 cells that had been previously treated with fractionated X-radiation were more resistant to single dose of X-radiation treatment than compared with cells that were not treated with fractionated radiation (See chapter 3). It is this observation that led to the rest of the work mentioned throughout this dissertation. The following chapters address the issue of whether radioresistance in our colorectal cancer model is preexisting or induced by the fractionation X-radiation treatment and to identify the mechanisms that may be responsible for the radioresponse of these cells.

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Qutob, S. S., Liu, Q. Y., McNamee. J. P. and Ng, C. E., The generation and characterization of human colorectal tumour clones with heterogeneous radiosensitivities to study the mechanisms of increased radioresistance following fractionated X-radiation treatment. *Int J Radiat Biol.*

3. ABSTRACT

Human colorectal tumour (HCT116) cells that had been previously treated with fractionated X-radiation (2 Gy X 10 daily fractions) were significantly more resistant to subsequent treatment with single doses of X-radiation (XR) relative to those that had not been exposed to fractionated irradiation. Our objectives were to determine whether the surviving subpopulation of radioresistant cells was due to the selection of a radioresistant subpopulation by, or to an inducible radiation response (IRR) to, the fractionated irradiation and if an observed IRR to a prior fractionated XR treatment is directly correlated with inherent XR resistance of the clone. To accomplish these objectives, we isolated several sister clones, with a wide range of responses to XR, from the parental, non-irradiated HCT116 cells by limited dilution. These clones included: (a) HCT116-Clone2, which was resistant to both fractionated and single dose XR relative to the parental HCT116 cells, (b) HCT116-CloneK, which was sensitive to both fractionated and single dose XR relative to the parental cells, (c) many clones, as typified by HCT116-Clone10, which responded similarly to both fractionated and single dose XR as the parental cells, and (d) HCT116-Clone4 which, interestingly, was resistant to fractionated, but not single dose, XR. There was no significant difference in survival following graded single doses of XR given to HCT116-Clone2 cells that had either been

previously treated with fractionated XR or not previously treated. Similar results were obtained with HCT116-Clone10 cells. In addition, there was no difference in survival between HCT116-Clone4 cells given a challenge single dose of XR regardless of whether the cells received, or did not receive, a previous priming dose of XR 24 hours earlier. There was no difference in results whether HCT116-Clone4 cells were treated in the exponential or plateau phase of growth. Although, HCT116-CloneK cells showed a possible IRR, the increase in clonogenic survival at 2 Gy (SF_2) for HCT116-CloneK cells that had previously seen fractionated radiation treatment was insufficient to suggest that these cells could not have been selected for by a series of 2 Gy fractions. We conclude that: (a) the uncloned, non-irradiated parental population of HCT116 cells is inherently heterogeneous in its response to XR, (b) the resistant subpopulation of cells surviving a previous fractionated irradiation exposure was likely due to the selection of a radioresistant subpopulation (likely from HCT116-Clone2-like cells) rather than to an adaptive response, (c) the radiation-sensitive, but not radiation-resistant, clone manifested a potential IRR, and (d) clonal heterogeneity of cellular response to XR, although widely acknowledged to occur *in vivo*, may also be important when evaluating the response of tumour cell populations grown as monolayer cultures *in vitro*.

3.1 INTRODUCTION

Radiation, used alone, as an adjunct to surgery, or in combination with chemotherapeutic drugs (termed chemoradiation), is widely applied in cancer therapy. In fact, chemoradiation has recently become very important in the treatment of various tumour types (e.g. advanced cervical, esophageal, rectal, lung, gastric). Clinically, X-radiation (XR) is usually fractionated (e.g. 2 Gy daily doses) and seldom delivered as single doses to avoid normal tissue morbidity. Our preliminary experiments demonstrated that human colorectal tumour (HCT116) cells given ten consecutive daily doses of 2 Gy XR *in vitro* were more resistant to a subsequent challenge with single doses of XR relative to HCT116 cells that had not been previously similarly treated with fractionated XR (Qutob *et al.*, 2004). Because of the obvious potentially negative clinical implications of an adaptive response (Bartel-Friedrich *et al.*, 1999; Chatterjee *et al.*, 1998; Ikushima, 1999; Iyer and Lehnert, 2002a; Iyer and Lehnert, 2002b; Mosse *et al.*, 2000; Russell *et al.*, 1995; Sasaki *et al.*, 2002) to fractionated XR treatment, we further evaluated the reason for the detection, and/or emergence, of a resistant subpopulation of HCT116 cells following the fractionated irradiation.

Previously, it has been widely acknowledged that tumour cells when grown, and treated, as solid tumours *in vivo* demonstrate a heterogeneous response to XR (Aabo, 1996). Several past studies had suggested that failure of some human carcinomas (human colon carcinoma) to respond to physical treatment modalities can be due to preexisting resistant subpopulations (Leith *et al.*, 1982; Wang *et al.*, 1999). Factors responsible for

affecting XR response *in vitro* and *in vivo* include both genetic (i.e. clonal heterogeneity) (Leith *et al.*, 1984) or epigenetic (e.g. related to tumour microenvironment, of which, hypoxia is a well known factor (Vaupel *et al.*, 2001). Other specific factors that may affect radioresponse may include chromosomal integrity, redox capability and DNA repair capacity (ap Rhys and Bohr, 1996; Kojima *et al.*, 1999; Leith *et al.*, 1982; Rothkamm *et al.*, 2003).

It is known that many cell-types possess the capacity to adapt to toxicity introduced by chronic exposure to chemical carcinogens and chemotherapeutic agents, by modulating the rate of uptake, excretion, or permeability as a means of decreasing the concentration of these toxins to vital cellular systems (Simon and Schindler, 1994). A number of investigations also provide evidence of adaptation in cell-types exposed to ionizing radiation (Flores *et al.*, 1996; Ikushima *et al.*, 1996; Ishii and Watanabe, 1996; Shadley and Wiencke, 1989). An inducible radiation response (IRR), which often increases cellular tolerance of very low doses of X- or γ-radiation, is well known and is referred to as the "radioadaptive response" (Dolling *et al.*, 2000; Ikushima, 1999; Iyer and Lehnert, 2002b; Olivieri *et al.*, 1984; Raaphorst *et al.*, 2000; Sasaki *et al.*, 2002; Skov, 1999; Wolff, 1998). In general, the radioadaptive response describes the ability of a priming dose of radiation to cause increased cellular resistance to a subsequent radiation exposure (Skov, 1999). Olivieri *et al.*, (1984) was the first to demonstrate induced-radioresistance *in vitro* in human blood lymphocytes pre-exposed to low priming doses of radiation which became less susceptible to chromatid aberrations induced by a subsequent high challenge dose of XR (Olivieri *et al.*, 1984). The radioadaptive response

was also shown to occur *in vivo* in blood lymphocytes extracted from individuals who were chronically exposed to levels of radiation higher than background. The mechanism responsible for conferring radioresistance to lymphocytes taken from these individuals was hypothesized to be a result of an induction of DNA repair (Gourabi and Mozdarani, 1998). In another published report, Iyer and Lehnert (2002) had speculated that the mechanism of radioadaption was a result of the presence of a transient transmissible factor or factors within cells and that levels of these factors may also determine the variability of radioadaptive response. They had developed this hypothesis based on the observation that normal human lung fibroblasts (HFL-1) previously treated with supernatants taken from low dose (1 cGy) gamma-irradiated HFL-1 cells, developed an increase in clonogenic survival following a challenge dose of 2 and 4 Gy. Furthermore, the radioadaptation exhibited by the HFL-1 cells, treated with the low dose irradiated HFL-1 supernatants, was associated with an increase in intracellular reactive oxygen species (ROS), increased reducing capacity and levels of the DNA repair protein, AP-endonuclease thus giving evidence of the presence of communicable factors that are responsible for determining cellular radiosensitivity (Iyer and Lehnert, 2002b).

However, not all reports show evidence of the radioadaptive response (Bosi and Olivieri, 1989; Hain *et al.*, 1992). Mortazavi *et al.*, (2003) suggested that the lack of the induction of radioresistance but the variability in radioresponse exhibited between cell lines or even individuals may not be attributed to these transient physiological factors but rather to some permanent constitutional predisposition (Mortazavi *et al.*, 2003). Based on this assumption, it is reasonable to suggest the possible existence of cell clones with

heterogeneous XR responses within a non-treated parental cell population that can lead to the selection of radioresistant clones when given a challenge dose of ionizing radiation. The rationale for investigating the development of radioresistance whether by adaptation or genetic inclination is of particular importance in the clinic since the presence of either radioadaption of tumour cells or tumour cells with pre-existing heterogeneous radiosensitivities can direct the type of treatment modality. Hence, the objectives of this study were: (a) to characterize radioresistance within a tumour cell population following fractionated XR and to determine whether this was due to an IRR to, or the selection of radiation-resistant cells by the treatment, and (b) to determine whether an observed IRR to a prior XR challenge in closely-related sister clone of human colorectal tumour (HCT116) cells is directly correlated with the inherent XR resistance of the clone.

3.2 MATERIALS AND METHODS

3.2.1 *Cell lines and culture:*

Human colorectal tumour (HCT116) cells, obtainable from the ATCC, were generously donated from Dr. Vogelstein (Waldman *et al.*, 1995). We cultured the cells in DMEM/ Ham's F12 1:1 mix containing 15 mM Hepes (Wisent) supplemented with 10% fetal bovine serum (Wisent) in a humidified atmosphere of 95% air, 5% CO^2 at 37 °C. We established clones from the non-irradiated parental cell line by limited dilution and used four of these clones (2, K, 4 and 10) for these studies. Cells/clones were grown in the exponential phase at the start of the treatment (X- radiation, drugs). The latter was achieved by seeding $2x10^5$ cells into a 25 cm^2 flask on day 0 and using these cells for experimentation on day 3. Fractionated X-radiation (XR) treated cell cultures were originally plated at the above-mentioned cell density and received fresh media changes every 3 days over the 10-day period. The plated cell numbers used for acute radiation sensitivity determination after fractionated XR treatment were similar to the non-treated-acute experiments. Additionally, surviving fractions determined during the fractionated XR were based on the plating efficiency from the non-treated cells at day 0.

3.2.2 *Isolation of Clones:*

HCT116 cells to be cloned were trypsinized and serially diluted in complete medium in 24 well, flat bottom Falcon MULTIWELL^{tm} tissue culture plates (Becton

Dickinson, Lincoln Park, New Jersey). Wells with one single colony were identified by light microscopy and subsequently marked. The cells of the marked colonies were recovered under the microscope by scraping the cells from the bottom of the plate and aliquoting 5 ul with a pipette tip. They were subsequently plated in one well of a 6-well plate and then trypsinized after cells were grown to 70% confluence and used to seed a 25 cm^2 flask. The cells in each flask were cultured for approximately two weeks, after which, they were subcultured into 75 cm^2 flasks.

3.2.3 *Irradiation:*

The cells/clones were treated with various doses of X-irradiation at room temperature using a 250 kVp X-ray unit (Pantak, CT) at a dose-rate of 150 cGy/min. For example, to obtain the desired dose of 2 Gy the cells were irradiated for 1 min and 20 seconds. For fractionated X-irradiation, cells were given 2 Gy daily doses for up to 10 consecutive days and immediately returned to the incubator. To test whether the emergence of radioresistant cells occurs through a selection or adaptation by the fractionated ionizing radiation treatment, cells were exposed to fractionated X-irradiation (10 days x 2 Gy) and 2 days after the last irradiation point were subsequently given varying acute doses of acute (i.e. single, non-fractionated dose) XR. To examine whether radioresistance displayed by the HCT116-Clone4 cells had occurred through adaptation to fractionated IR exposure, HCT116-Clone4 cells were given various priming doses (0 to 4 Gy) of IR, allowed to incubate at 37°C for either 6 or 24 hours and then assessed for survival following a challenge dose of 8 Gy.

3.2.4 *Colony-forming assay:*

Immediately following either acute or fractionated treatment with XR, cells were rinsed twice with isotonic citrate saline, trypsinized (0.2% trypsin/2.5 mM EDTA for 5 min. at 37 ^{O}C) and counted with an electronic particle counter (Particle Data Inc., Elmhurst, IL). Cell suspensions were plated to yield about 50 colonies per 60 mm dish after 11 days at 37 ^{o}C in a humidified atmosphere of air containing 5 % CO_2. Cell culture dishes were stained with methylene blue and colonies containing more than 50 cells were scored to assess surviving fraction. Survival curves were normalized based on the corresponding plating efficiencies. Radiation survival curves were fitted with the linear quadratic model $S = \exp(-\alpha D - \beta D^2)$ using Sigma Plot software (SPSS).

3.2.5 *Alkaline comet assay:*

Exponential-phase cells were treated with XR of doses of 0, 0.25, 0.50, 1.0 and 2.0 Gy. Cells were trypsinized, counted, and diluted in culture medium to a concentration of 4 x 10^5 cells/ml. A 1:10 dilution in agarose of this cell suspension was made by mixing 50 µl of cell of the cell suspension with 450 µl of 1 % LMP agarose (Gibco BRL) in PBS (Sigma) at 40 ^{o}C in a 1 ml Eppendolf tube. For each dose point, 120 ml of this solution was aliquoted to an exposure well on a strip of Gelbond (Mandel) film prepared according to the protocol for alkaline comet assay (McNamee et al., 2002). Each experiment was performed five times and DNA damage was quantified using the ALKOMET v3.1 image analysis system (Richard Brancker Research Ltd, Ottawa,

Ontario). Tail length and moment were calculated and were plotted as a function of XR dose.

3.3 RESULTS

After fractionated treatment, HCT116 cells were collected and a single dose X-radiation (XR) survival assay was performed, and then compared with the acute survival curve obtained with cells that were not pre-treated with fractionated XR. Figure 1 showed an increase in survival of parental HCT116 cells following treatment to daily 2 Gy x 10 fractions compared to those that have not been previously exposed to fractionated XR. To determine whether this was due to an adaptive response to, or the selection of radiation-resistant cells by the previous fractionated radiation treatment, clones were selected from the non-irradiated parental cell population by limited dilution and subsequently irradiated. In figure 2, no significant morphological differences were observed between parental HCT116, HCT116-Clone10, and HCT116-CloneK whereas HCT116-Clone2 and HCT116-Clone4 cells had an elongated fusiform shape, consisting of multiple focal adhesions, which were distributed about the periphery of the cell. We have shown that following X-irradiation, HCT116-Clone2 was significantly resistant to both fractionated and single dose XR treatment relative to the parental cells (i.e. HCT116-Clone2 was a radioresistant clone) (figure 3a). HCT116-CloneK was more sensitive to both fractionated and single dose radiation treatments (i.e. radiosensitive clone) (figure 3b) and HCT116-Clone10 as well as nine other clones demonstrated radiosensitivity similar to the parental cell population (i.e. non-radioresistant or non-sensitive clones) (figure 3c). We have also isolated a clone (HCT116-Clone4) that responded similarly as HCT116-Clone2 to fractionated XR, in comparison to parental HCT116 cells, but displayed a

radiation response that was similar to parental HCT116 cells when exposed to single dose irradiations (Fig 4).

Figure 1. Survival curves demonstrating the presence of radioresistant HCT116 cells following fractionated X-radiation exposures. Cells were collected and plated following daily 2 Gy irradiations for ten days and measured for survival following exposure to varying single acute doses of X-radiation. Each point represents the mean and standard error of four independent experiments; within each experiment, each point was obtained by averaging 2-3 dishes.

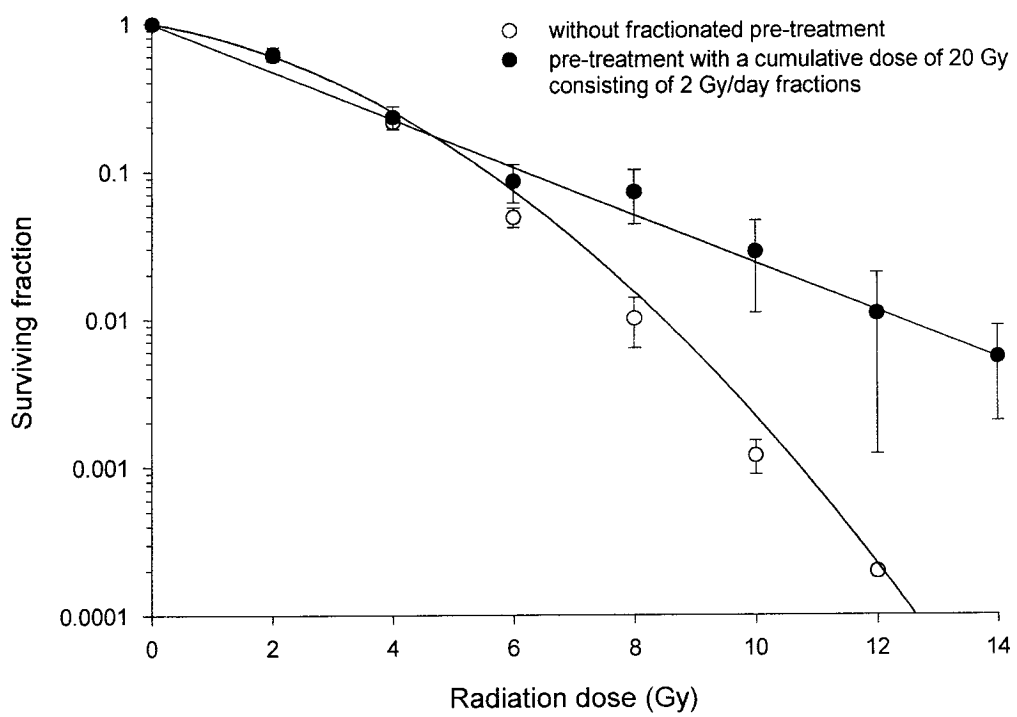

without fractionated pre-treatment
pre-treatment with a cumulative dose of 20 Gy consisting of 2 Gy/day fractions
Surviving fraction
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
12
14
Radiation dose (Gy)

Figure 2. Cell morphology profiles of: (a) parental HCT116, (b) HCT116-Clone10, (c) HCT116-CloneK, (d) HCT116-Clone2, and (e) HCT116-Clone4. Cells were examined by phase contrast microscopy at 200× magnification.

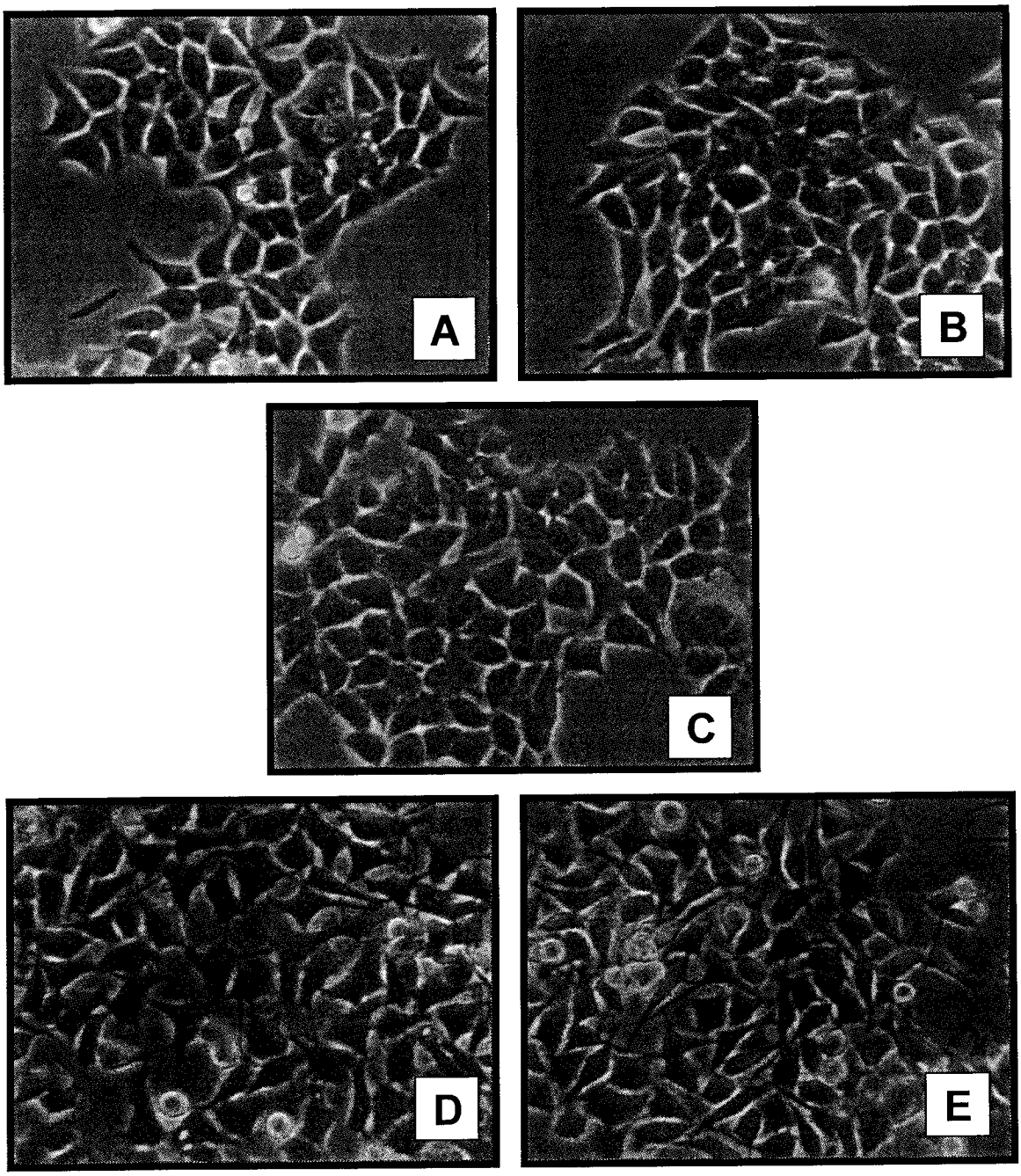

A
B
C
D
E

Figure 3a. Radiation response of HCT116-Clone2 vs. parental HCT116. Clonogenic surviving fraction of HCT116-Clone2 and the parental HCT 116 cells following either fractionated (closed symbols) or acute X-radiation treatments (open symbols). For the fractionated experiments, the x-axis represents the total accumulative X-radiation dose received by the cells. For parental HCT116 cells, each point represents the mean and standard error of four independent experiments for single doses of radiation treatment and two for fractionated treatment. For HCT116-Clone2 cells, each point represents the mean and standard error of three independent experiments for single and fractionated doses of radiation treatment; within each experiment, each point was obtained by averaging 2-3 dishes.

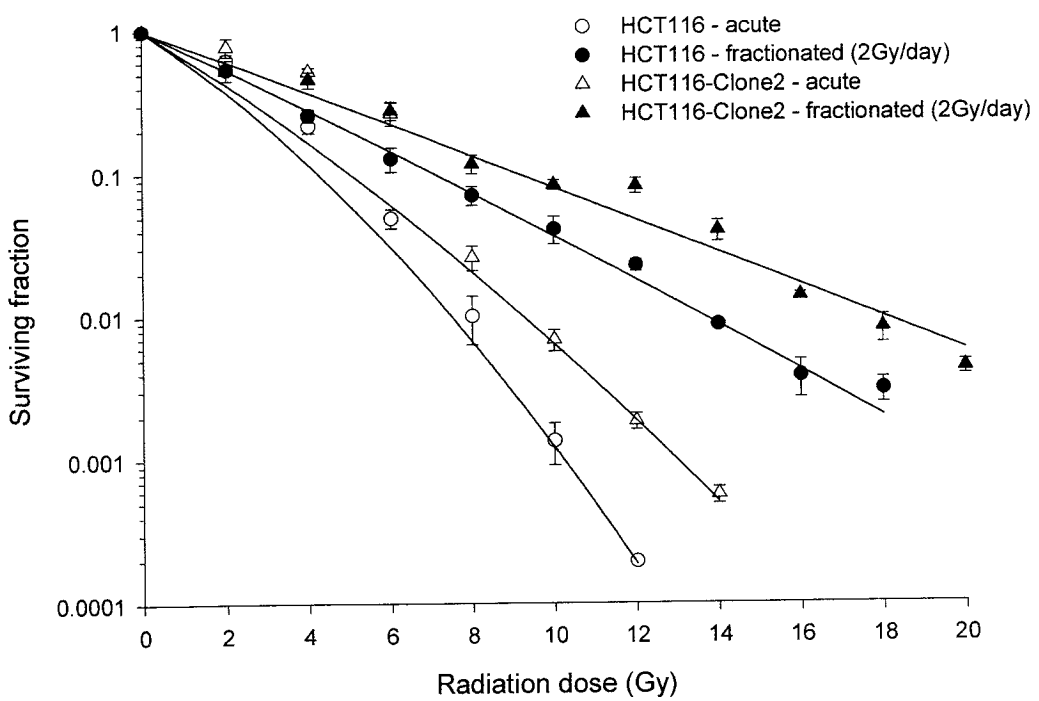

HCT116 - acute
HCT116 - fractionated (2Gy/day)
HCT116-Clone2 - acute
HCT116-Clone2 - fractionated (2Gy/day)
Surviving fraction
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
12
14
16
18
20
Radiation dose (Gy)

Figure 3b. Radiation response of HCT116-CloneK vs. parental HCT116. Clonogenic surviving fraction of HCT116-CloneK and the parental HCT 116 cells following either fractionated (closed symbols) or acute X-radiation treatments (open symbols). For the fractionated experiments, the x-axis represents the total accumulative X-radiation dose received by the cells. For HCT116-CloneK cells, each point represents the mean and standard error of two independent experiments for single and fractionated doses of radiation treatment; within each experiment, each point was obtained by averaging 2-3 dishes.

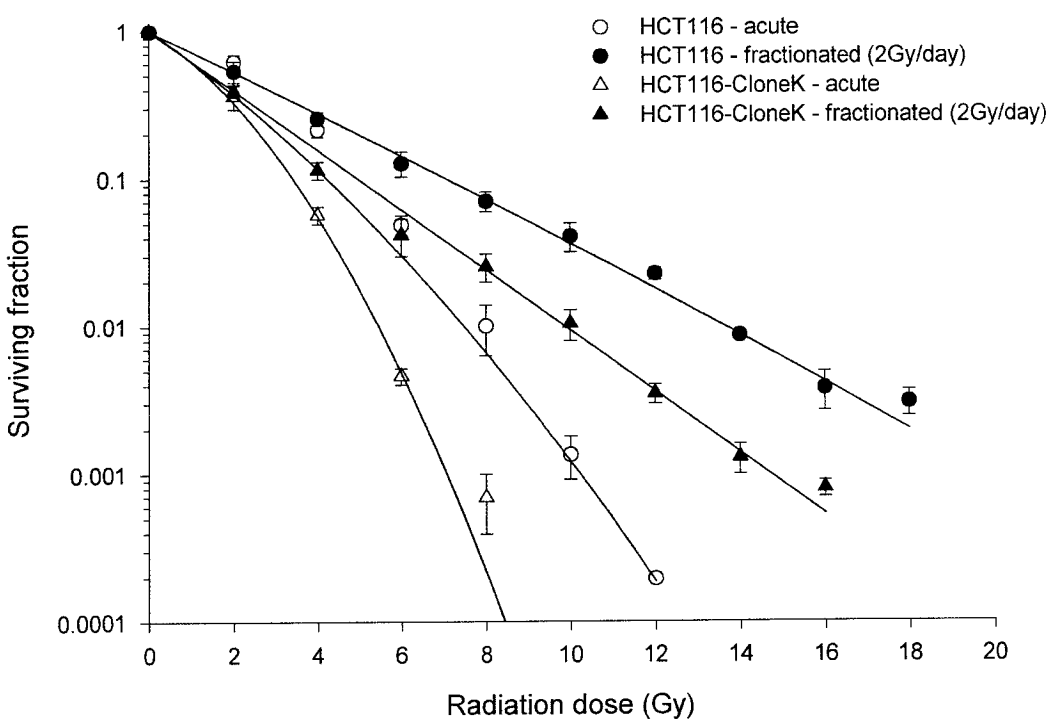

HCT116 - acute
HCT116 - fractionated (2Gy/day)
HCT116-CloneK - acute
HCT116-CloneK - fractionated (2Gy/day)
Surviving fraction
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
12
14
16
18
20
Radiation dose (Gy)

Figure 3c. Radiation response of HCT116-Clone10 vs. parental HCT116. Clonogenic surviving fraction of HCT116-Clone10 and the parental HCT 116 cells following either fractionated (closed symbols) or acute X-radiation treatments (open symbols). For the fractionated experiments, the x-axis represents the total accumulative X-radiation dose received by the cells. For HCT116-Clone10 cells, each point represents the mean and standard error of three independent experiments for single and fractionated doses of radiation treatment; within each experiment, each point was obtained by averaging 2-3 dishes.

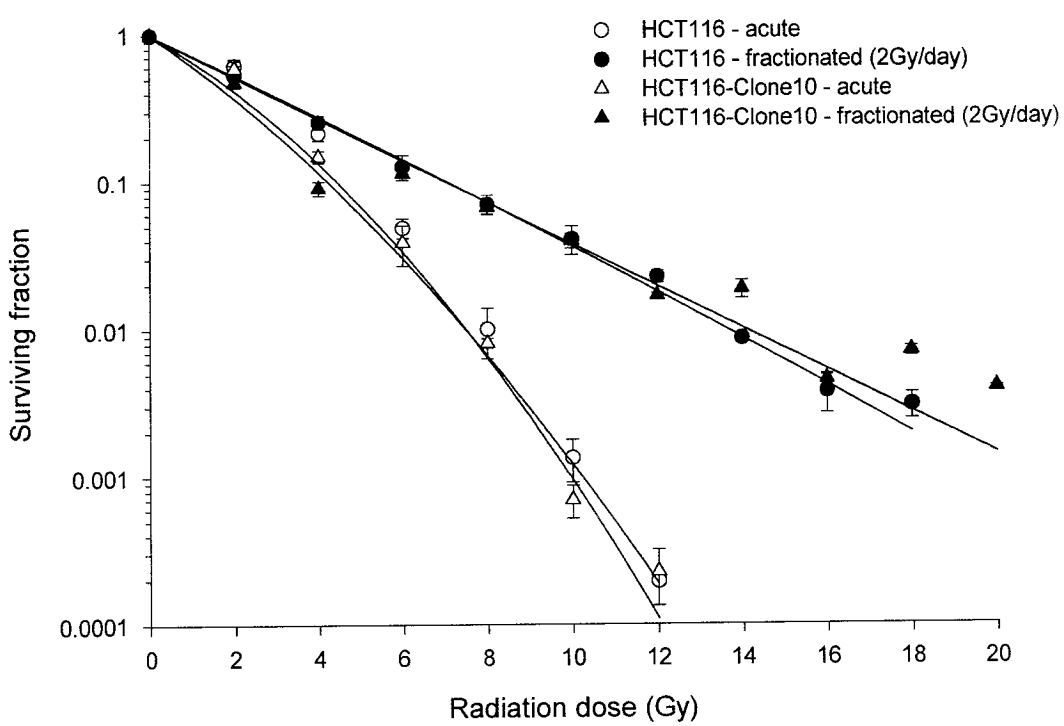
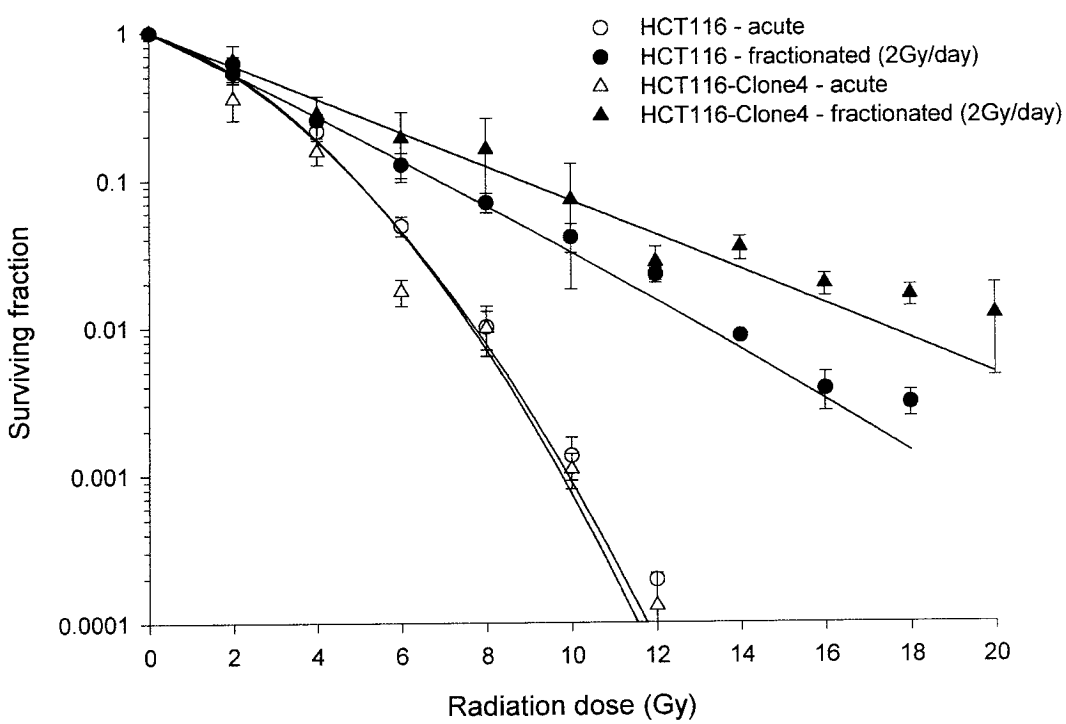

HCT116 - acute
HCT116 - fractionated (2Gy/day)
HCT116-Clone10 - acute
HCT116-Clone10 - fractionated (2Gy/day)
Surviving fraction
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
12
14
16
18
20
Radiation dose (Gy)

Figure. 4. Radiation response of HCT116-Clone4 vs. parental HCT116. Clonogenic surviving fraction of HCT116-Clone4 and the parental HCT 116 cells following either fractionated (closed symbols) or acute X-radiation treatments (open symbols). For the fractionated experiments, the x-axis represents the total accumulative X-radiation dose received by the cells. For HCT116-Clone4 cells, each point represents the mean and standard error of three independent experiments for single and fractionated doses of radiation treatment; within each experiment, each point was obtained by averaging 2-3 dishes.


HCT116 - acute
HCT116 - fractionated (2Gy/day)
HCT116-Clone4 - acute
HCT116-Clone4 - fractionated (2Gy/day)
Surviving fraction
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
12
14
16
18
20
Radiation dose (Gy)

The alkaline comet assay revealed no significant differences in either the tail length (figure 5a) or tail moment (figure 5b), as determined by repeated measures ANOVA, in the amount of DNA damage (including single-stranded breaks, double-stranded breaks, and alkali-labile damage) between any of the clones at each dose group of 0, 0.25, 0.50, 1.0 and 2.0 Gy.

To address the issue whether fractionated treatment can alter radiosensitivity, the clones were given a single dose of XR treatment following a previous exposure to fractionated XR. The curves generated were then compared with the curve developed from cells that were not treated with fractionated XR. Figure 6a shows that HCT116-Clone10 regardless of whether it had received any previous fractionated XR treatment responded to single dose X-irradiations similarly to the non-fractionated parental HCT116 cell line.

Figure 6b shows that HCT116-Clone2 cells responded similarly regardless of whether it had received any previous fractionated XR. Interestingly, HCT116-CloneK exhibited a significantly higher clonogenic survival following fractionated XR (one-tailed Student's *t*-test, P-values < 0.05 for doses > 4 Gy), suggesting that there might have been some adaptive responses to the fractionated XR (figure 6c).

Figure 5a. Data from the alkaline comet assay showing DNA damage (single-strand breaks, double-strand breaks and alkali-labile) expressed as normalized tail length after acute XR doses of 0, 0.25, 0.5, 1, and 2 Gy. Each point represents the average of five experiments.

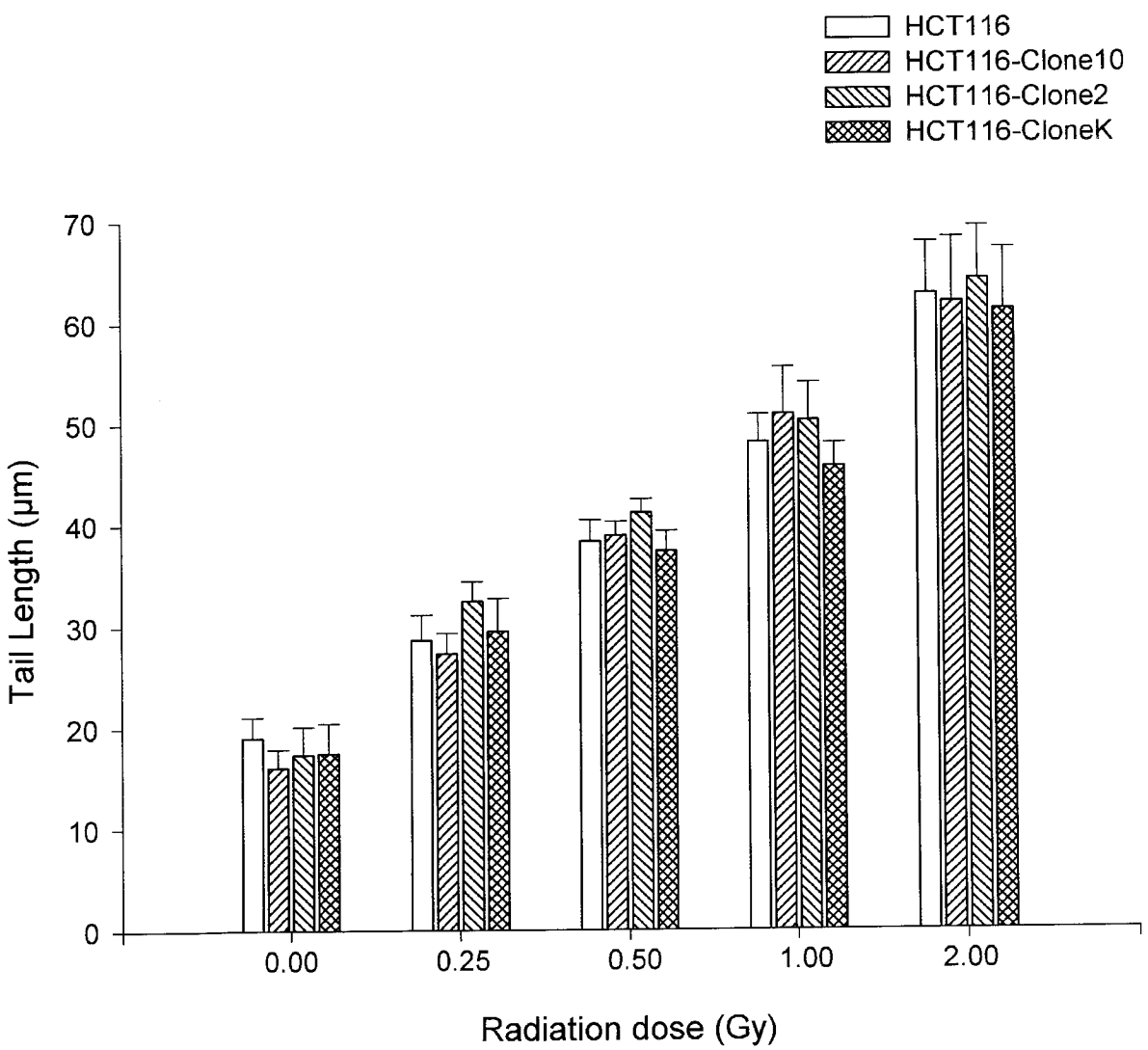

HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
Tail Length (μm)
0
10
20
30
40
50
60
70
0.00
0.25
0.50
1.00
2.00
Radiation dose (Gy)

Figure 5b. Data from the alkaline comet assay showing DNA damage (single-strand breaks, double-strand breaks and alkali-labile) expressed as normalized tail moment after acute XR doses of 0, 0.25, 0.5, 1, and 2 Gy. Each point represents the average of five experiments.

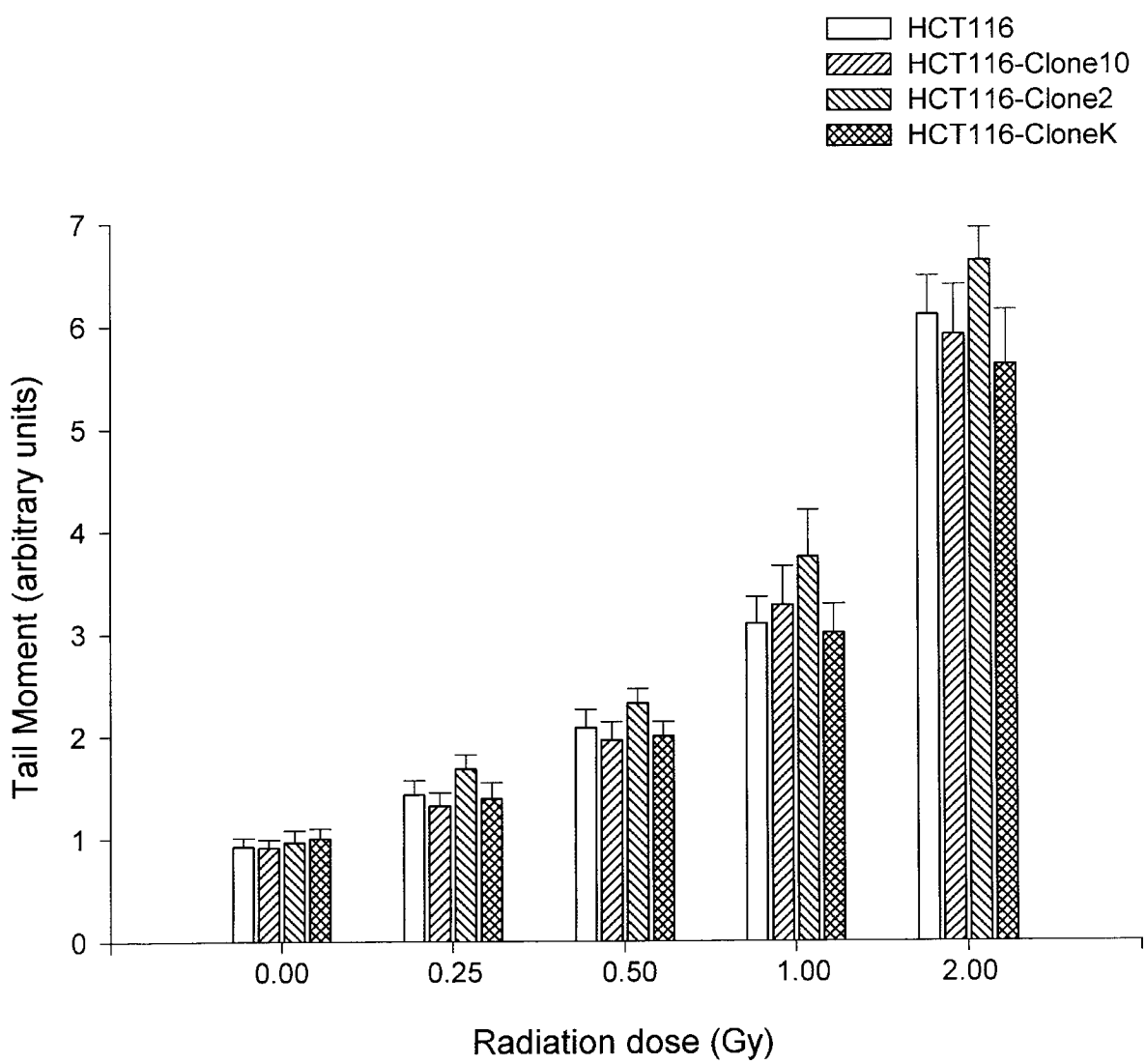

HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
Tail Moment (arbitrary units)
0
1
2
3
4
5
6
7
0.00
0.25
0.50
1.00
2.00
Radiation dose (Gy)

Figure 6a. Single dose survival curves of parental HCT116 and HCT116-Clone10 cells with or without previous exposure to (10 x 2 Gy) fractionated X-radiation treatment. HCT116-Clone10 and parental HCT116 cells were collected and plated following either fractionated (closed symbols) or sham treatment (open symbols) and measured for survival following exposure to varying single acute doses of IR. For parental HCT116 cells, each point represents the mean and standard error of four independent experiments for single and fractionated doses of radiation treatment. For HCT116-Clone10 cells, each point represents the mean and standard error of one independent experiment for single and pretreatment with fractionated doses of radiation treatment; within each experiment, each point was obtained by averaging 2-3 dishes.

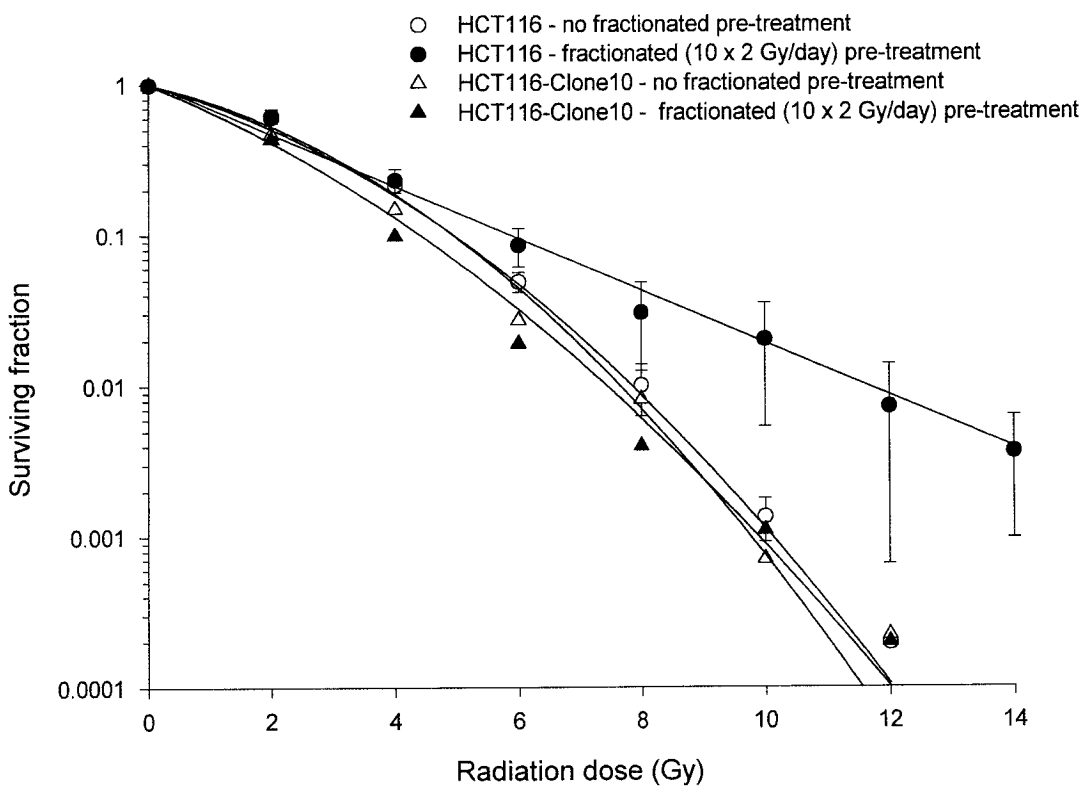

HCT116 - no fractionated pre-treatment
HCT116 - fractionated (10 x 2 Gy/day) pre-treatment
HCT116-Clone10 - no fractionated pre-treatment
HCT116-Clone10 - fractionated (10 x 2 Gy/day) pre-treatment
Surviving fraction
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
12
14
Radiation dose (Gy)

Figure 6b. Single dose survival curves of parental HCT116 and HCT116-Clone2 cells with or without previous exposure to (10 x 2 Gy) fractionated X-radiation treatment. HCT116-Clone2 and parental HCT116 cells were collected and plated following either fractionated (closed symbols) or sham treatment (open symbols) and measured for survival following exposure to varying single acute doses of IR. For HCT116-Clone2 cells, each point represents the mean and standard error of two independent experiments for single and pretreatment with fractionated doses of radiation treatment; within each experiment, each point was obtained by averaging 2-3 dishes.

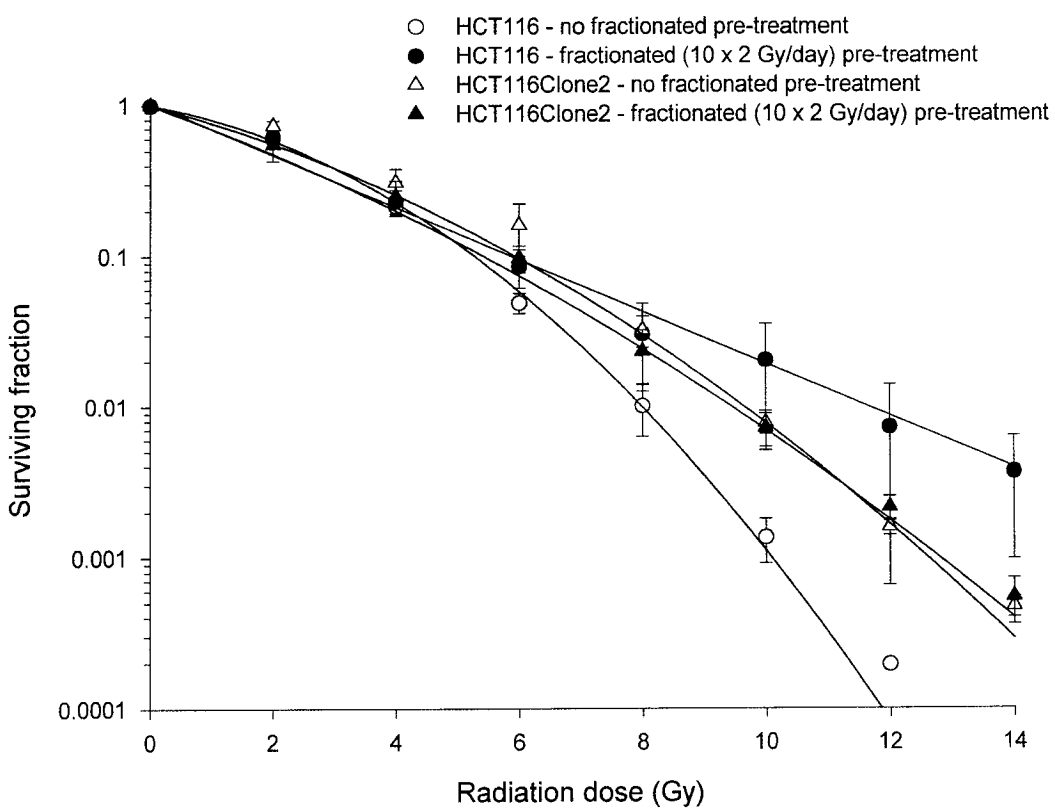
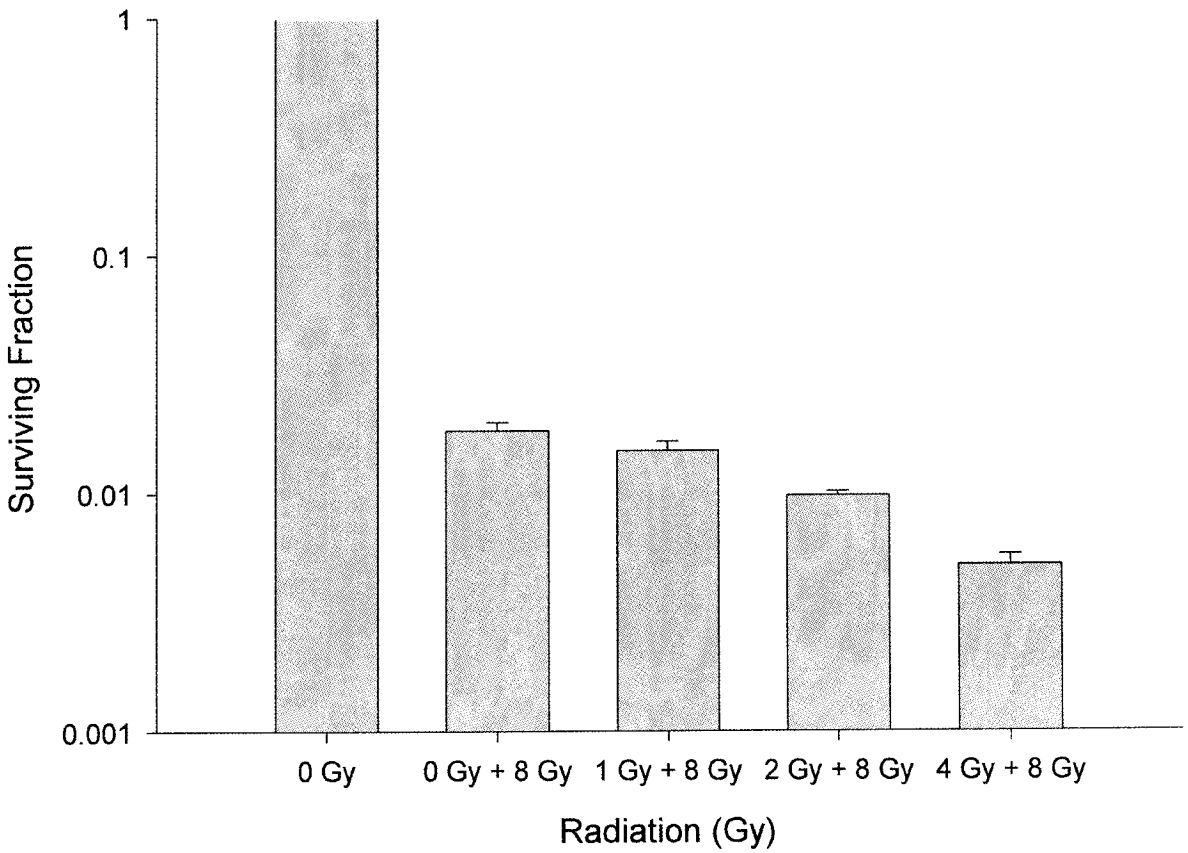

HCT116 - no fractionated pre-treatment
HCT116 - fractionated (10 x 2 Gy/day) pre-treatment
HCT116Clone2 - no fractionated pre-treatment
HCT116Clone2 - fractionated (10 x 2 Gy/day) pre-treatment
Surviving fraction
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
12
14
Radiation dose (Gy)

Figure 6c. Single dose survival curves of parental HCT116 and HCT116-CloneK cells with or without previous exposure to (10 x 2 Gy) fractionated X-radiation treatment. HCT116-CloneK and parental HCT116 cells were collected and plated following either fractionated (closed symbols) or sham treatment (open symbols) and measured for survival following exposure to varying single acute doses of IR. For HCT116-CloneK cells, each point represents the mean and standard error of three independent experiments for single doses of radiation and four experiments with pretreatment of fractionated doses of radiation treatment; within each experiment, each point was obtained by averaging 2-3 dishes.

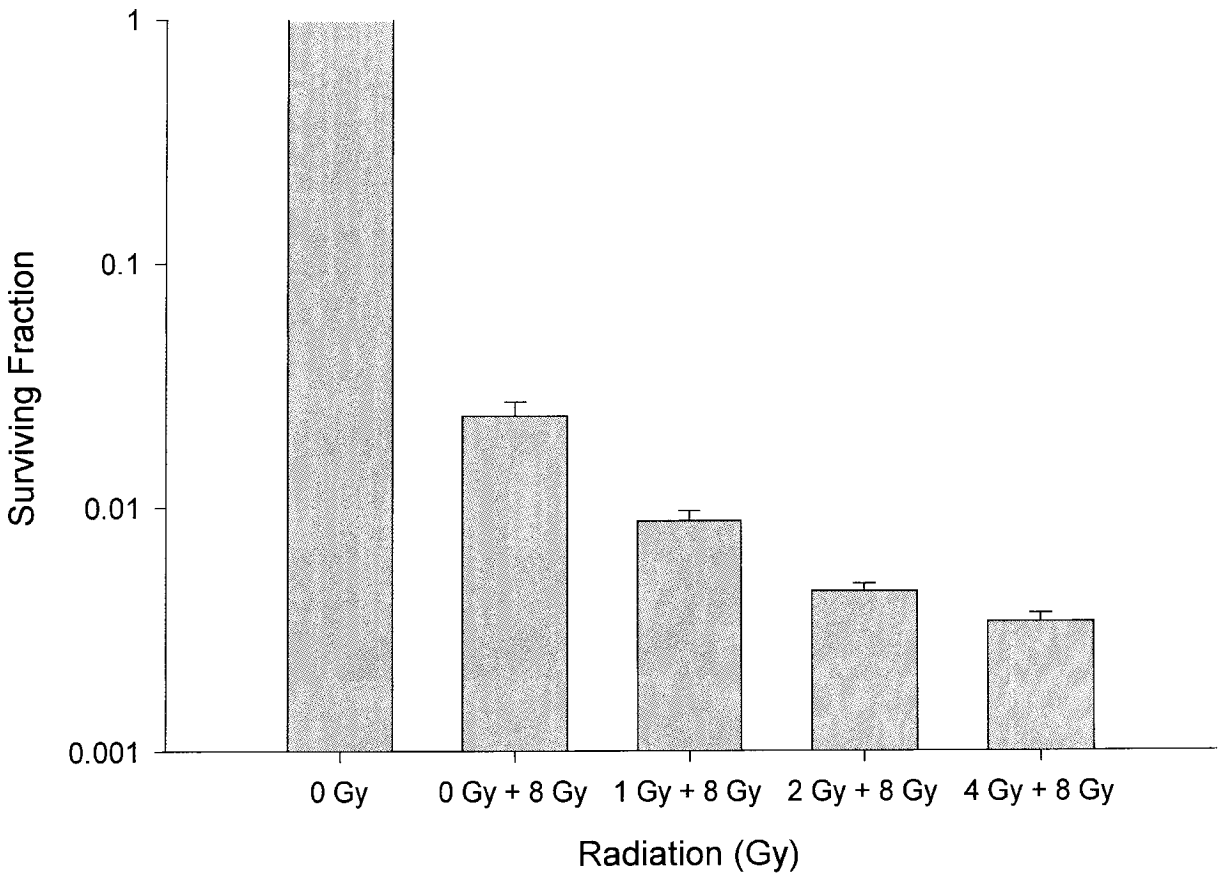
Figures 7a and 7b had shown that priming doses of either 1, 2, or 4 Gy and 24h before a challenging dose of 8 Gy failed to induce radioresistance within HCT116-Clone4 cells in either plateau or exponential phase of the cell cycle, respectively.

Figure 7a. Lack of adaptive response (i.e. increase in surviving fraction) in HCT116-Clone4 plateau phase cells. Plateau phase HCT116-Clone4 cells were given varying priming doses of IR, allowed to incubate for 24 hours and then measured for survival following a higher challenge dose of IR. Each point represents the mean and standard error of triplicate dishes within each experiment.



24 hour incubation at 37°C between radiation treatments

Figure 7b. Lack of adaptive response (i.e. increase in surviving fraction) in HCT116-Clone4 exponential phase cells. Exponential phase HCT116-Clone4 cells were given varying priming doses of IR, allowed to incubate for 6 hours and then measured for survival following a higher challenge dose of IR. Each point represents the mean and standard error of triplicate dishes within each experiment.



6 hour incubation at 37°C between radiation treatments

3.4 DISCUSSION

Heterogeneous radioresponses have been well documented to occur *in vivo*, however, its importance has not been extensively examined in determining radioresponses of tumour cell lines. Figure 1 clearly demonstrates the presence of a radioresistant subpopulation of cells from a well-established parental HCT116 cell line following treatment with fractionated radiation. From this non-treated parental HCT116 cell line, we have isolated by limited dilution a number of cell clones with varying radioresponses. Our study bears some resemblance to a 1996 study by *Britten et al.*, In his investigation, the radiosensitivity of cell lines derived from primary cultures of three carcinomas of the cervix were determined and single-cell clones were selected by limited dilution and subsequently sub-cultured. These clones were also determined to have heterogeneous radioresponses; a number of which were significantly different from the parental cell population (Britten *et al.*, 1996). Similarly, we have also provided evidence of heterogeneous radiosensitivities amongst clones taken from a well-established cell line. In this case, clones were selected from a non-treated parental cell population and their radioresponse was characterized by acute doses of radiation. The results of our data shows that the majority of clones selected, including HCT116-Clone10 and ten other clones, whether exposed to fractionated, or single acute doses of X-radiation (XR) demonstrated survival that was similar to the parental cell line not treated with fractionated XR (figure 3a). However, the sub-line HCT116-Clone2 (figure 3b) was shown to have a significantly increased survival when subjected to either of these treatments whereas HCT116-CloneK (figure 3c) had a significantly decreased survival

relative to the parental cell line. Also, we did not detect significant differences in intrinsic DNA damage among the clones following XR doses of up to 2.0 Gy, as determined by the comet assay, indicating no evidence of any intrinsic differences of DNA protective mechanisms between the clones or that the X-ray dose given wasn't high enough to distinguish these differences.

In contrast to Britten's study, the clones isolated in our study were not derived directly from excised tumours, which are known to be heterogenous in nature, but originated from a well-established cell line, normally understood to be of a homogenous composition, thus providing evidence of clonal variability even within a well-established cell line. Intriguingly, these clones not only differed in their radioresponses but are also heterogeneous in their morphology (figure 2). Tumour clonal variations in morphology are not unusual and has been documented by several researchers (Dexter *et al.*, 1978; Palmari *et al.*, 1997), however, only a small number of studies correlate these clonal morphological differences with heterogeneous intrinsic radiosensitivities. Several investigators did find that trypsin-induced contraction and rounding of spread cells causes enhanced radiosensitivity (Kapiszewska *et al.*, 1991; Reddy and Lange, 1991; Reddy *et al.*, 1989) but this might be explained by the elimination of cell-to-cell contact which is known in itself to cause an increase in the radiation response (Kwok and Sutherland, 1991). In another report, the authors associated radiosensitive A549 lung cancer cells stably transfected with hyperactive Integrin-linked kinase with changes in cell morphology signified by spindle-like cell shape with multiple focal adhesions (Cordes, 2004). Therefore, it is likely that a change in cell shape alone does not determine

radioresponsiveness but may be a direct consequence of clonal cytogenetic differences since we observed a similar cell shape in our most radioresistant clones and not our radiosensitive clone.

Tumour cell populations have been known to be more genetically unstable than comparable normal cells and that genetic instability, characterized by either the loss or gain of whole chromosomes, may contribute to the generation of heterogeneous subpopulations (Nowell, 1982). Genetic instability is a landmark in colorectal cancer, a process responsible for the development 80–85% of colorectal neoplasms (Ricciardiello and Bazzoli, 2002). We have found that our HCT116 colorectal clones was associated with a number of the metaphases displaying stable novel chromosomal abnormalities (Qutob *et al.*, 2004) that may be responsible for the observed varying radioresponses amongst the cell clones. Therefore, it is the sequential appearance and selection of more radioresistant cell subpopulations that may be responsible for defining and/or predicting the overall radiosensitivity of the parental cell line. For example, the enrichment of the HCT116 cell line with relatively more radioresistant cells (i.e. HCT116-Clone2, HCT116-Clone4) and elimination of the radiosensitive clones (i.e. HCT116-CloneK) may explain the detection of a radioresistant sub-population following fractionated radiation. This is important since the successful treatment of tumour tissue is dependent on removal of the radioresistant cells since it is these cells that will likely repopulate and provide complications for further treatment.

It is now well known that small, single doses of XR can elicit an adaptive response (often resulting in cells becoming more resistant to a subsequent XR exposure) (Skov, 1999). However, it is relatively less clear whether clinically relevant fractionated 2 Gy XR treatments can invoke a similar phenomena. To address this issue, we exposed the clones to fractionated XR prior to single acute doses to evaluate possible changes in radioresponsiveness. HCT116-Clone10 cells responded to single acute dose irradiations similarly to the non-treated parental HCT116 cell and fractionated XR treatment was unable to modulate that response. Additional, evidence of selection is provided by the HCT116-Clone2 since the clone also maintains its radiosensitivity regardless of its previous treatment and is radioresistant in comparison to the non-treated parental HCT116 cell line, demonstrating that radioresistance exhibited by HCT116-Clone2 was not induced by the fractionated XR.

Radioresistance was also demonstrated by HCT116-Clone4 but only when previously treated with fractionated XR indicating a possible radioadaptive response. However, priming doses of XR (0 to 4 Gy) failed to induce radioresistance in HCT116-Clone4 cells when subsequently challenged with a XR dose of 8 Gy, 24 hours after the first priming dose. It has been shown that the cell cycle can influence the degree of cellular radioresistance. For instance, most cells are relatively radioresistant in the plateau-phase of the cell-cycle and the reasons for this include the efficient repair of potentially lethal damage (PLD) and changes in the structure of the DNA and the accessibility of DNA repair enzymes to sites of DNA damage (Lisby *et al.*, 2003; Marples *et al.*, 2003). Therefore, we had performed the majority of our experiments in the

exponential phase of growth in all of our clones to maximally display differences in cellular radiosensitivity as well as to reduce cell–cell contact that has been shown to also influence the radiation response (Kwok and Sutherland, 1991). However, we show that priming doses of XR given to HCT116-Clone4 cells in either plateau or exponential phase of the cell cycle does not alter its radiosensitivity. No change in clonogenic survival was also witnessed in plateau-phase C3H 10T1/2 mouse fibroblast cells when an adapting dose of 0.1 to 1.5 Gy was administered but a reduction in the frequency of micronucleus formation and neoplastic transformation was shown following exposure to a challenge dose of 4 Gy (Azzam *et al.*, 1994). What our data suggests is that small doses of daily-fractionated irradiation over a long period of time may be required to induce an increase in radioresistance as witnessed with HCT116-Clone4 cells.

Interestingly, after fractionated irradiation the radiation-sensitive HCT116-CloneK, but not the radiation-resistant HCT116-Clone2, manifested a potential inducible radiation response (IRR) (figure 6c). This finding did not provide any evidence for a correlation between the magnitude of the IRR and the inherent radiation resistance of the treated tumour cells. Also, no significant difference was seen in the surviving fraction at 2 Gy (SF_2) for HCT116-CloneK that had or had not seen fractionated radiation treatment (P-values > 0.05, one-tailed Student's *t*-test – figure 6c) suggesting that an adaptive response, if any, in HCT116-CloneK would still have been insufficient to result in the selection of HCT116-CloneK-like cells following a series of 2 Gy fractions. An alternative explanation is that changes had occurred in the expression of radiation response genes by the introduction of spontaneous mutations due to a defect in mismatch

repair attributed with this cell line (Mure and Rossman, 2001; Robinson *et al.*, 2003) or that the radiation exposure induced genomic instability (Kronenberg, 1994) along with the relative hypersensitivity of the HCT116-CloneK cell population may allow for the presence of less radiosensitive cell clones within the HCT116-CloneK subline which have been selected for by the fractionated XR treatment.

Various studies also give evidence for selection in the development of radioresistance. For example, Wang *et al*, (1999) had generated a set of cell clones with varying radiosensitivities by subjecting the non-irradiated parent glioma cell line (U-251 MG-Ho) to a chemical mutagen. These clones were found to maintain their relative radiosensitivities for at least 30 cell culture passages giving proof of a stable trait that was not induced by the radiation treatment. Interestingly, the most radiosensitive and radioresistant clones did not differ significantly in either the induction or repair of radiation-induced DNA double-strand breaks, however, potentially lethal damage repair was greater in the radioresistant clone than in the radiosensitive clone. Furthermore, radiation-induced apoptosis were found to be similar in both clones (Wang *et al.*, 1999) thereby suggesting that radioresistance does not require suppression of cell death mechanisms.

In another study, a radioresistant cell strain had been obtained after prolonged exposure to x-rays for 7 months (2 Gy per day, 5 days per week) from the human fibrosarcoma HT 1080 (Wei *et al.*, 1998). Similar to the previous study, the ability of the radioresistant cells to rejoin DNA double-strand breaks, the cell cycle distribution and the

amount of apoptosis after irradiation was no different than the parental strain. However, the investigators had suggested that the radioresistance may have been caused by radiation-induced cell mutation but did not address the possibility that the resistant cells had been selected for by the repeated irradiations. Therefore, a possible problem with the majority of these studies is that clones are selected for after radiation treatment that will only provide information on gene expression for a particular radiation response and may not distinguish gene expressions associated on how a cell will response to radiation. We, on the other hand, have shown that our radioresistant clone did not develop due to the fractionated radiation treatment since it was selected from a non-treated parental cell line and the treatment did not affect its radiosensitivity. It is possible that differences in the radiosensitivities of the cell clones may be a result of variations in the stage or duration of the cell cycle phases amongst the clones. However, fractionated therapy, known to cause shifts to more radiosensitive phases of the cell cycle, did not alter sensitivity of clones 2 and 10, but may play a role in the inducible resistance response in clone 4, since it was observed only following fractionation and not when given a single priming dose prior to a challenge dose. Moreover, the apparent lack of an inducible resistance response in clones 2 and 10 that had seen fractionated radiation treatment, allowed us to conclude that the radioresistant subpopulation in figure one arose from selection (likely of HCT116-Clone2-like cells). However, the increase in radiation resistance displayed by the radiation-sensitive clone (HCT116-CloneK), following fractionated radiation treatment, may have been due to an inducible radiation response or a result of asynchronous cycling cells within the HCT116-CloneK parental population thereby selecting more resistant s-phase cells. To determine this possibility, cell cycle analysis on HCT116-CloneK

following fractionated radiotherapy is required to determine its involvement on HCT116-CloneK induced resistance.

The isolation of cell clones with varying radioresponses within a non-treated *in vitro* cell culture and the effect of these clones on the total radioresponsiveness of the cell line following a fractionated XR regiment, similar to the regiments used in radiotherapy, can provide some insight of the possible mechanisms involved in treatment failure and therefore would have possible implications for clinical radiotherapy. To achieve this endpoint, similar experiments will need to be performed on a number of different tumour cell lines and their clone sublines. As well, the genes responsible for radiosensitivity of these cells will also need to be examined since tumour genomes have been shown to be determined by selective pressures for alterations that promote cell survival (Snijders *et al.*, 2003). Currently, we have identified in the HCT116 clones a number of genes with no apparent link to DNA repair that may play a role for determining radiosensitivity (Qutob, S.S., Ng, C.E., submitted for publication – see chapter 5) however more investigations are required in other tumour models, in order, to identify the genes involved in providing cells with a survival advantage to ionizing radiation.

3.5 REFERENCES

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Qutob, S. S., Multani, A. S., Pathak, S., Feng, Y., Kendal, W. S., and Ng, C. E., Comparison of the X-radiation, drug and ultraviolet-radiation responses of clones isolated from a human colorectal tumour cell line. *Radiat. Res.*

4. ABSTRACT

We isolated several clones with a wide range of responses to X-radiation from an unirradiated human colorectal (HCT 116) tumour cell line. The responses of one of these clones (HCT116-Clone10) and nine other clones to either fractionated, or acute (i.e. single, non-fractionated doses), X-radiation in vitro was similar to that of the parental cell line. By contrast, following the same types of treatment, another clone (HCT116-Clone2) manifested a significantly increased survival whereas a third clone (HCT116-CloneK) manifested a significantly decreased survival relative to the parental cell line. This suggested that they were, respectively, a radioresistant and a radiosensitive clone. All three clones (clones 2, 10, K) retained their tumourigenic phenotype and formed tumours in nude mice. G-banding studies confirmed that they were of human origin and were derived from the same parental cell line. The metaphases of HCT116-Clone2 demonstrated features commonly associated with genomic instability (i.e., mitotic catastrophe including chromosome and chromatid breaks, dicentric and additional nonclonal markers). Quantitative fluorescence in situ hybridization (Q-FISH) analysis data failed to demonstrate any apparent correlation between the expression of radiosensitivity and relative telomere content of these three clones. Interestingly, HCT116-CloneK was the most resistant to several chemotherapeutic drugs

(topotecan, camptothecin, etoposide and cisplatin) with diverse mechanisms of action. Also, there were no significant differences in the survival of all three clones following treatment with UV-radiation. Because of the non-overlap among the relative sensitivities of these clones to X-radiation, chemotherapeutic drugs and UV-radiation, these clones may be useful models for evaluating the genetic basis of the response of human tumour cells to these treatment agents both in vitro and in vivo.

4.1 INTRODUCTION

Established tumour or normal cell lines growing in vitro have been widely used as preclinical models for investigating the responses of these cells to diverse treatment agents and for understanding the cellular mechanisms that are associated with these responses. However, it is known that clonal heterogeneity exists even among the same type of cells growing under well-controlled conditions in vitro. Thus sister clones derived from the same parental cell line have been shown to possess distinguishable biological properties or demonstrate dissimilar responses to various types of treatment, excluding X-radiation (1-4). Interestingly, although there are many reports of variation in X-radiation response among sister clones derived from previously X-radiated cells (5-9), this is less frequently found among sister clones isolated from an unirradiated parental cell population (10, 11).

The aims of this study were, therefore, to examine whether we could isolate clones with a wide range of responses to X-radiation from an untreated human colorectal tumour cell line and, if we could, to further characterize their biological properties. We describe in

this paper three clones that have vastly different responses to X-radiation given either as fractionated or single doses, to four common chemotherapeutic drugs, and to UV-radiation. These clones retained their tumourigenicity in vivo suggesting that they may be useful preclinical models for evaluating the response to various treatment agents in vitro as well as in vivo.

4.2 MATERIALS AND METHODS

4.2.1 *Cell line and culture:* Human colorectal tumour (HCT 116) cells, also available from the American Type Culture Collection (ATCC), were a gift from Dr. Vogelstein (12). We cultured the cells in DMEM/Ham's F12 1:1 mix (Wisent, St. Bruno, QC) supplemented with 10% fetal bovine serum (Wisent, St. Bruno, QC) and 15 mM Hepes (Boehringer Mannheim) in a humidified atmosphere of 95 % air, 5 % CO_2 at 37 °C. We isolated clones from the unirradiated parental cell line by limited dilution and used three of these clones (2, K and 10) for these studies. Clonal cell cultures were in the exponential phase of growth at the start of all treatments (X-radiation, drugs and UV-radiation). The latter was achieved by seeding $2x10^5$ cells into a 25 cm^2 flask on day 0 and using these cells for experimentation on day 3. Fractionated X-radiation treated cell cultures were originally plated at the above-mentioned cell density and received fresh media changes every 3 days over the 10-day period. The plated cell numbers used for acute radiation sensitivity determination after fractionated X-radiation treatment were similar to the untreated-acute experiments. Additionally, surviving fractions determined during the fractionated X-radiation were based on the plating efficiency

from the untreated cells at day 0.

4.2.2 *Growth of tumours in vivo:* We injected $1x10^7$ cells of the respective clone into each 8-week-old CD-1 nude mouse (Wilmington, MA) and waited 7-10 days before taking the first measurement of tumour volume with vernier calipers. We calculated the tumour volume (V) from the equation V = 0.52 x length x width x height; measurements were made orthogonally. The animal care committee of the U. of Ottawa approved the protocol for the use of laboratory mice.

4.2.3 *X-radiation, drug exposures and UV-irradiation:* We treated the cells/clones with various doses of acute (i.e. single, non-fractionated dose) X-radiation at room temperature. We used a 250 kVp X-ray unit (Pantak, CT) at a dose-rate of 150 cGy/min. For fractionated X-radiation, cells were given 2 Gy daily doses for up to 10 consecutive days and immediately returned to the incubator.

Camptothecin (CPT) and etoposide (VP-16), both purchased from Sigma (St. Louis, MO), were dissolved in DMSO as previously described (13). We reconstituted Topotecan (Hycamptin, SmithKline Beecham, Oakville, ON) (TPT) in sterile water as per the manufacturer's instructions and further diluted with PBS to the required concentrations. Cisplatin (CDDP) was obtained as a clinical formulation in 0.9 % saline (Faulding (Canada) Inc., Montreal, QC). All drug exposures were for 1 h at 37 °C.

For UV-irradiation, cells were exposed to a germicidal bulb emitting predominantly at 254 nm. The fluence (1 J/m^2/sec.) was determined before each experiment using a UVX

radiometer (UVP Inc., San Gabriel, CA).

4.2.4 *Clonogenic assay:* Immediately following treatment with X-radiation, drug (CPT, TPT, VP-16 or CDDP) or UV-radiation, the cells were rinsed twice with isotonic citrate saline solution. The cells were then trypsinized (0.2% trypsin/2.5 mM EDTA for 5 min at 37 ^{o}C) and counted with an electronic particle counter (Particle Data Inc., Elmhurst, IL). We plated cell suspensions to yield about 50 colonies per 60 mm dish after 14 days in a humidified atmosphere of air containing 5 % CO_2. Three dishes were plated for each dose in every experiment and we carried out 2-3 independent experiments for each clone for each treatment. We stained dishes with methylene blue and colonies containing more than 50 cells were scored to assess surviving fraction. We fitted radiation survival curves with the linear quadratic model $S = \exp(-\alpha D - \beta D^2)$ (14). We tested statistical significance using the one-tailed Student's t-test.

4.2.5 *Giemsa (G)-banding of chromosomes:* We cultured approximately 10^5 cells/ml in T-75 flasks. After 48 hours, cells in the exponential phase of growth were treated with colcemid (0.04 μg/ml) for 30 min, followed by a hypotonic solution of potassium chloride (0.06 M) for 20 min at room temperature. We then harvested the cells and fixed them in a mixture of methanol and acetic acid (3:1 by volume). Air-dried slides were made, and the optimally-aged slides used for G-banding analyzes following standard techniques described previously (15).

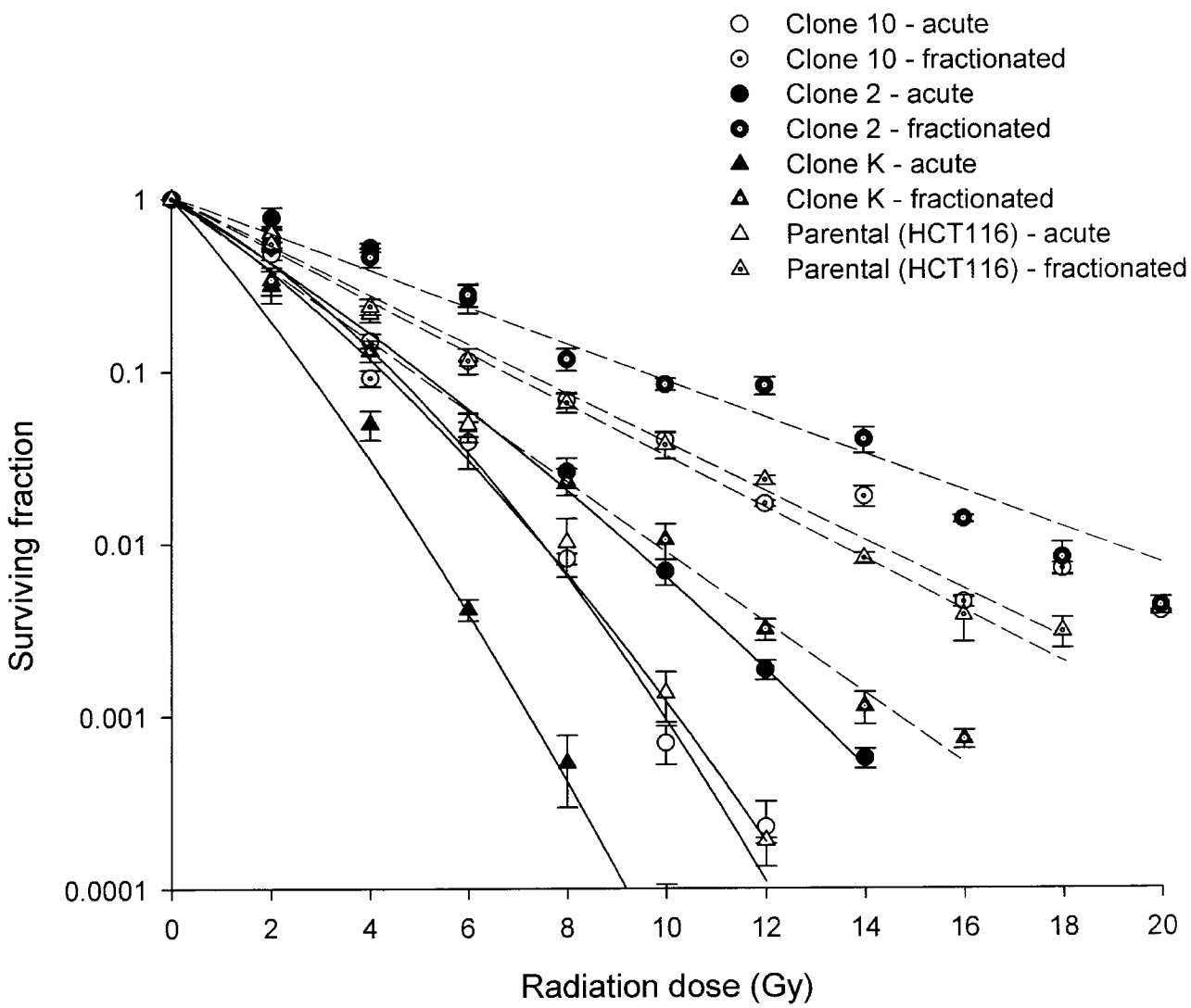
4.2.6 *Q-FISH analysis of telomeric DNA:* Telomeric DNA was evaluated using a Cy3-labeled peptide nucleic acid (PNA) telomere probe (Dako Corporation, Carpinteria, CA), following the manufacturer's protocol with slight modifications (16). FISH preparations were examined with a Nikon photomicroscope equipped with a UV-2A filter for DAPI and a Cy3 filter (Omega Optical Inc., Brattleboro, VT). Photomicrographs of FISH preparations were taken with a cooled charged couple-device (CCD) camera and the images analysed with a software package (Universal Imaging Co., Westchester, PA). We assessed the ratio of telomeric area as a percent of total interphase nuclear area with this software package on a minimum of 50-100 cells and calculated the mean and standard deviation of these values.

4.3 RESULTS

Because of the clinical relevance of fractionated X-radiation treatments, we evaluated the responses of several clones to fractionated (given as a series of daily 2 Gy) as well as acute treatments. There were no significant differences in the response of HCT116-Clone10 (Fig. 1) as well as nine other clones (data not shown) to either type of treatment relative to the parental cell line. By contrast, HCT116-Clone2 cells were clearly more resistant to both types of treatment relative to the parental cell line (Fig. 1). HCT116-CloneK cells, however, were significantly more sensitive to both these types of treatment (Fig. 1). Plating efficiencies for the three clones and the parental cells in these experiments were similar (53-55%). In addition, we had screened at least 15 other clones and found that they showed no significant differences in their responses to acute X-radiation relative to the parental cells (i.e.

Fig. 1 Clonogenic surviving fraction of the three clones and the parental HCT 116 cells following either fractionated (dashed lines) or acute X-radiation treatments (solid lines). For the fractionated experiments, the x-axis represents the total accumulative X-radiation dose received by the cells. Parental cells and HCT116-Clone10 cells displayed overlapping X-radiation responses. HCT116-Clone2 was more resistant whereas HCT116-CloneK was more sensitive to either fractionated or acute X-radiation relative to the parental cells. Each point represents the mean and S.E. of three independent experiments; within each experiment, each point was obtained by averaging 2-3 dishes.

Figure 1.


Clone 10 - acute
Clone 10 - fractionated
Clone 2 - acute
Clone 2 - fractionated
Clone K - acute
Clone K - fractionated
Parental (HCT116) - acute
Parental (HCT116) - fractionated
Surviving fraction
1
0.1
0.01
0.001
0.0001
0
2
4
6
8
10
12
14
16
18
20
Radiation dose (Gy)

fractionated treatment not done).

Table 1 shows the values of the surviving fraction at 2 Gy (SF_2) as well as the "α" and "β" components of the linear quadratic fits to the survival curves depicted in Fig. 1. As expected, HCT116-CloneK had the lowest SF_2 for both fractionated and acute X-radiation treatments. The difference between the SF_2 values of HCT116-CloneK and the parental population for acute, but not fractionated, treatment was statistically significant ($p<0.04$). There were no statistical significance between the SF_2 values of the parental population and either HCT116-Clone2 or HCT116-Clone10 for both types of treatment. For acute X-radiation, HCT116-CloneK had the highest, and HCT116-Clone2 the lowest, "β" value (Table 1). The difference in "β" values between the parental cells and HCT116-Clone2 was statistically significant ($p<0.02$) whereas the difference between the parental and HCT116-CloneK was marginally significant ($p<0.053$). There were, however, no significant differences between the "β" values of the parental cells and any of the clones for fractionated treatment. The "α" value of HCT116-CloneK was significantly larger than that of the parental cells for fractionated, but not acute, X-radiation ($p<0.04$, Table 1). The differences in "α" values between the parental cells and those of the other two clones were not significantly different for any treatment ($p<0.05$).

Fig. 2a shows the growth rates of these three clones in vitro. HCT116-Clone2 had a faster rate of growth (i.e. shorter doubling time 28.7 h, higher saturation density) in vitro relative to the other two clones and the parental cells (HCT116-Clone10 doubling time of 67.5 h, HCT116-CloneK of 45 h and parental cells of 33 h). There were no significant differences, however, in the size distribution of these clones (data not shown).

Table 1.

Cell line	Treatment	α	β	SF_2
Parental	*Acute*	0.212 ± 0.061	0.050 ± 0.010	0.625 ± 0.065
	Fractionated	0.316 ± 0.043	0.002 ± 0.003	0.540 ± 0.090
Clone 10	*Acute*	0.334 ± 0.042	0.031 ± 0.004	0.590 ± 0.075
	Fractionated	0.337 ± 0.031	0.001 ± 0.001	0.478 ± 0.038
Clone 2	*Acute*	0.333 ± 0.050	0.016 ± 0.004	0.660 ± 0.010
	Fractionated	0.230 ± 0.030	0.001 ± 0.002	0.530 ± 0.028
Clone K	*Acute*	0.394 ± 0.065	0.081 ± 0.007	0.370 ± 0.070
	Fractionated	0.507 ± 0.038	0.001 ± 0.001	0.395 ± 0.035

All errors are the standard errors of the mean

Fig. 2 Growth curves of the three clones and the parental cells. HCT116-Clone2 had the shortest doubling time of 28.7 h. HCT116-Clone10 had the longest doubling time of 67.5 h. HCT116-CloneK and parental cells had comparable doubling times of 45 h and 33 h, respectively. Each point represents the mean and standard error (S.E.): (a) of three dishes in vitro, and (b) of 3-5 tumours (i.e. one tumour per mouse) in vivo. Dashed line represents the growth curve of the parental HCT 116 cell line under our (a) in vitro, or (b) in vivo experimental conditions.

Figure 2a.

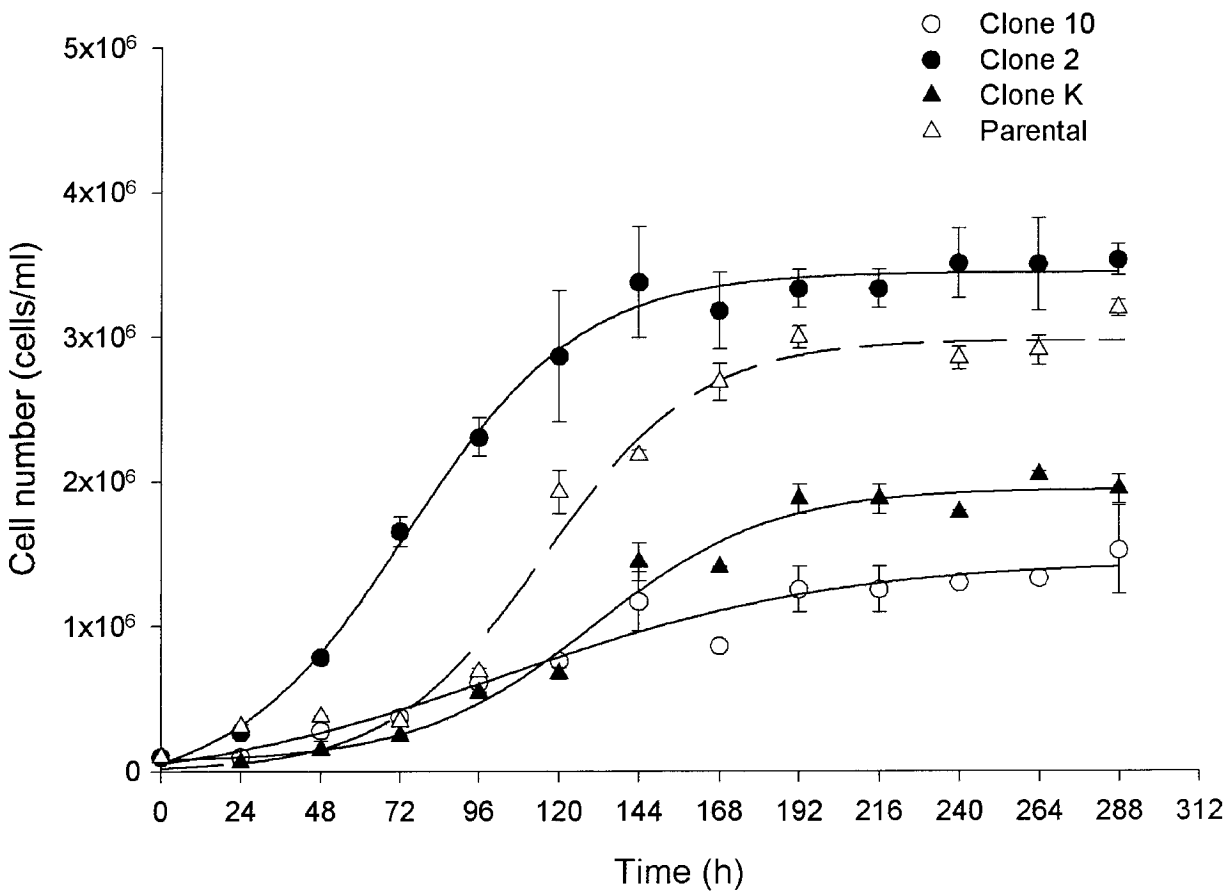
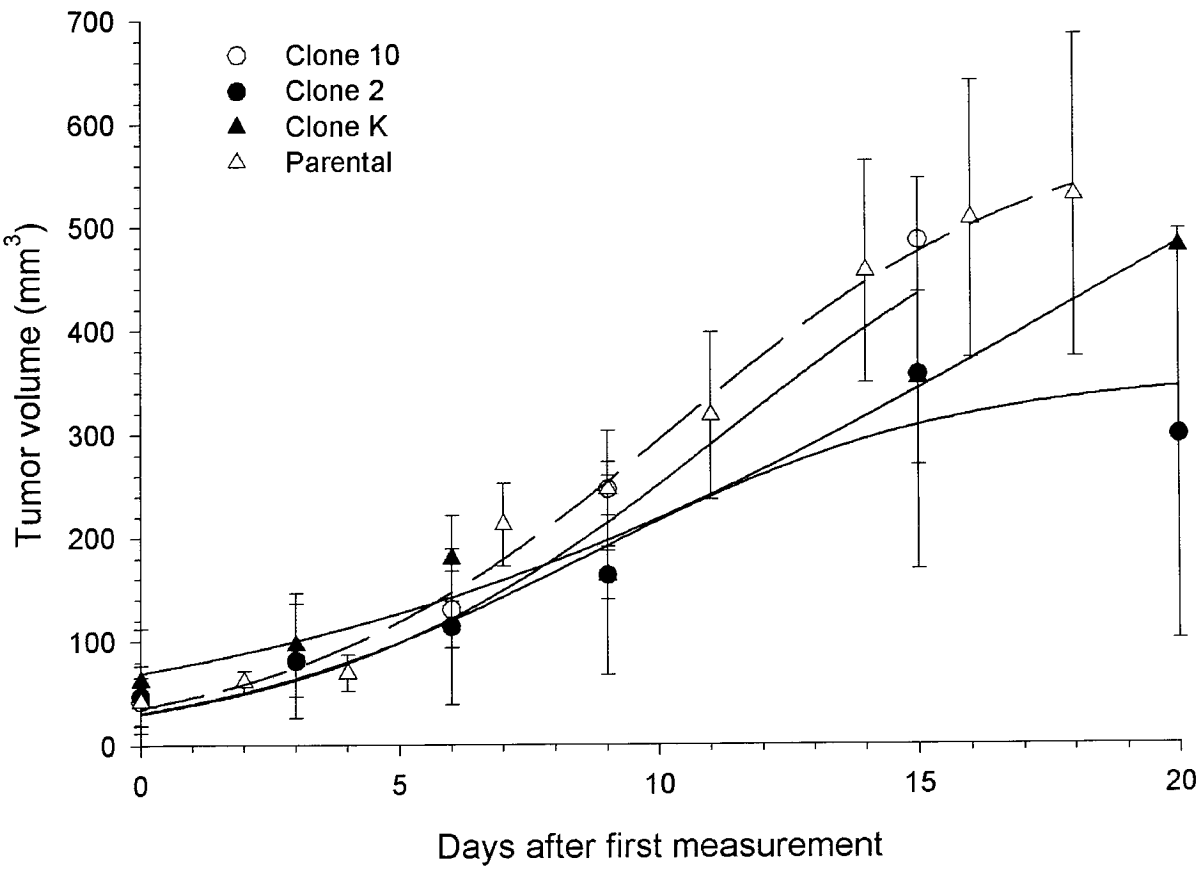

Clone 10
Clone 2
Clone K
Parental
5x10^6
4x10^6
3x10^6
2x10^6
1x10^6
0
Cell number (cells/ml)
0
24
48
72
96
120
144
168
192
216
240
264
288
312
Time (h)

Figure 2b.


Clone 10
Clone 2
Clone K
Parental
Tumor volume (mm3)
Days after first measurement
0
100
200
300
400
500
600
700
0
5
10
15
20

Morphologically, clones 10 and K appeared more similar to each other than to HCT116-Clone2. All three clones retained their tumourigenicity and formed tumours in nude mice. It was not possible for us to determine whether there were any significant differences in their growth rates relative to each other or relative to the parental untreated HCT 116 cell line due to the small number of mice (i.e. 3-5 per group) that were used in these experiments and the relatively large variation in tumour volumes (Fig. 2b). However, there were tumour takes in all the mice that were injected with the three clones.

The chromosome numbers in 20 metaphases each of both clones 2 and 10 cells ranged from 39 to 46 with a peak of 44 chromosomes (Figures 3a I. and 3b). By contrast, the chromosome numbers in 25 metaphases of HCT116-CloneK cells ranged between 45 and 92 with a peak at 45 chromosomes (Fig. 3c). The chromosome numbers in 25 metaphase spreads of parental HCT 116 cells ranged from 39-92 with a peak of 45 chromosomes (data not shown). Therefore, the modal chromosome numbers in these three clones and the parental cell line were 44-45. Fig. 3a II shows the marker chromosomes from the parental HCT116 cell line. The metaphases of HCT116-Clone2 and parental HCT 116 cells showed features commonly associated with genomic instability. These features included cells with mitotic catastrophe (chromosome and chromatid breaks, dicentric and additional nonclonal markers). In addition, clones 2 and 10 each had single copies of chromosome 22 but HCT116-CloneK had two copies of this particular chromosome. However, the parental line showed two types of metaphases, one with single and other with two copies of chromosome 22.

Clones 2 and 10 had at least six identical marker chromosomes, M1 - M6, as

Fig. 3 Giemsa-banded karyotypes, showing both structural and numerical chromosome abnormalities. of: (a) HCT116-Clone10 (note: lower panel, II, shows the marker chromosomes from the parental HCT116 cells for comparison), (b) HCT116-Clone2, and (c) HCT116-CloneK. The clonal marker chromosomes (M1 to M6) from an additional metaphase spread are arranged on the bottom row of each clone. The marker and the characteristic chromosome no. 9 from two parental metaphases are arranged on the bottom two rows of figure 3a panel II. Selected examples of mitotic catastrophe from HCT116-Clone2 are also shown on the bottom row.

Figure 3a.

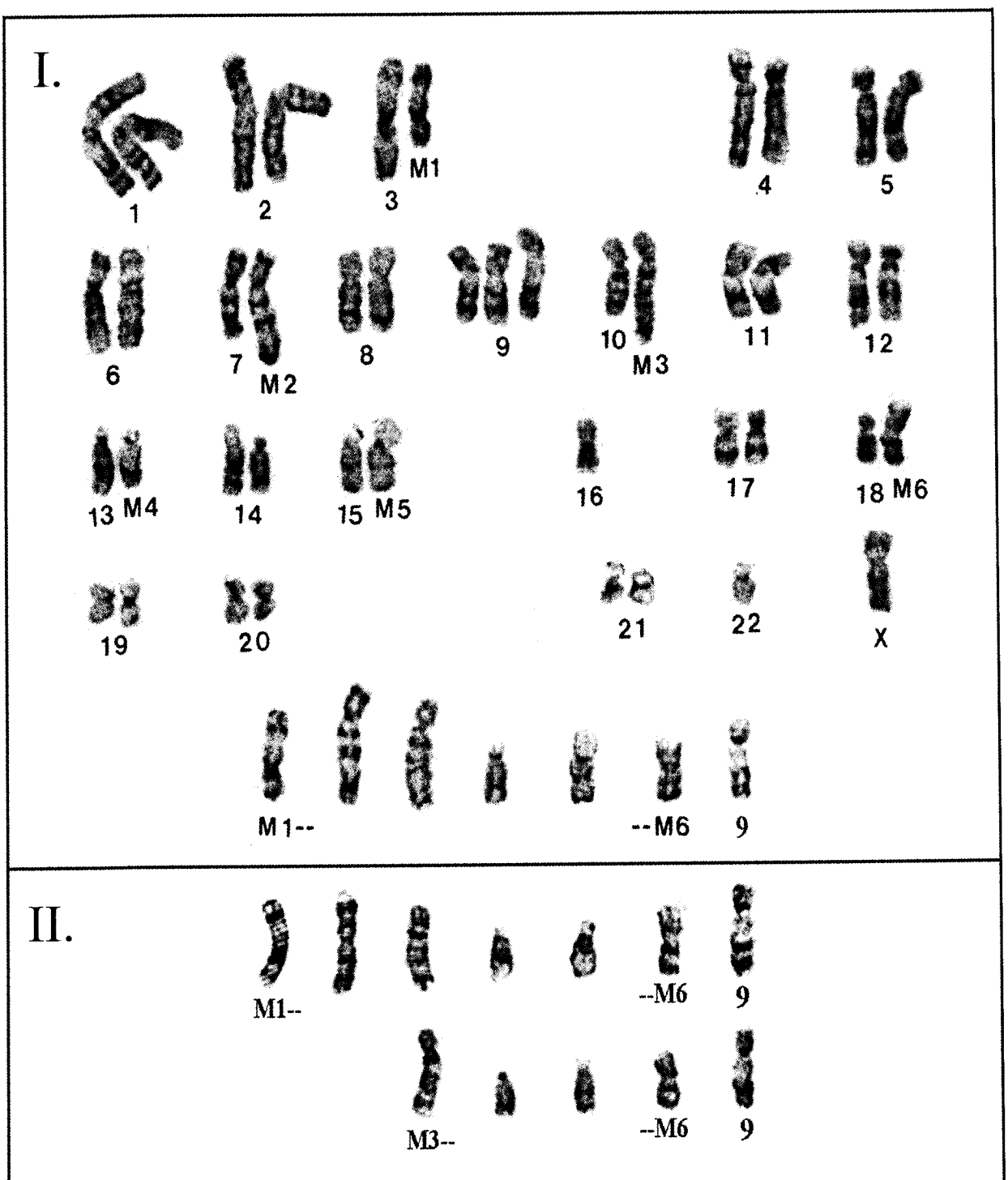

I.
1
2
3
M1
4
5
6
7
M2
8
9
10
M3
11
12
13
M4
14
15
M5
16
17
18
M6
19
20
21
22
X
M1--
--M6
9
II.
M1--
--M6
9
M3--
--M6
9

Figure 3b.

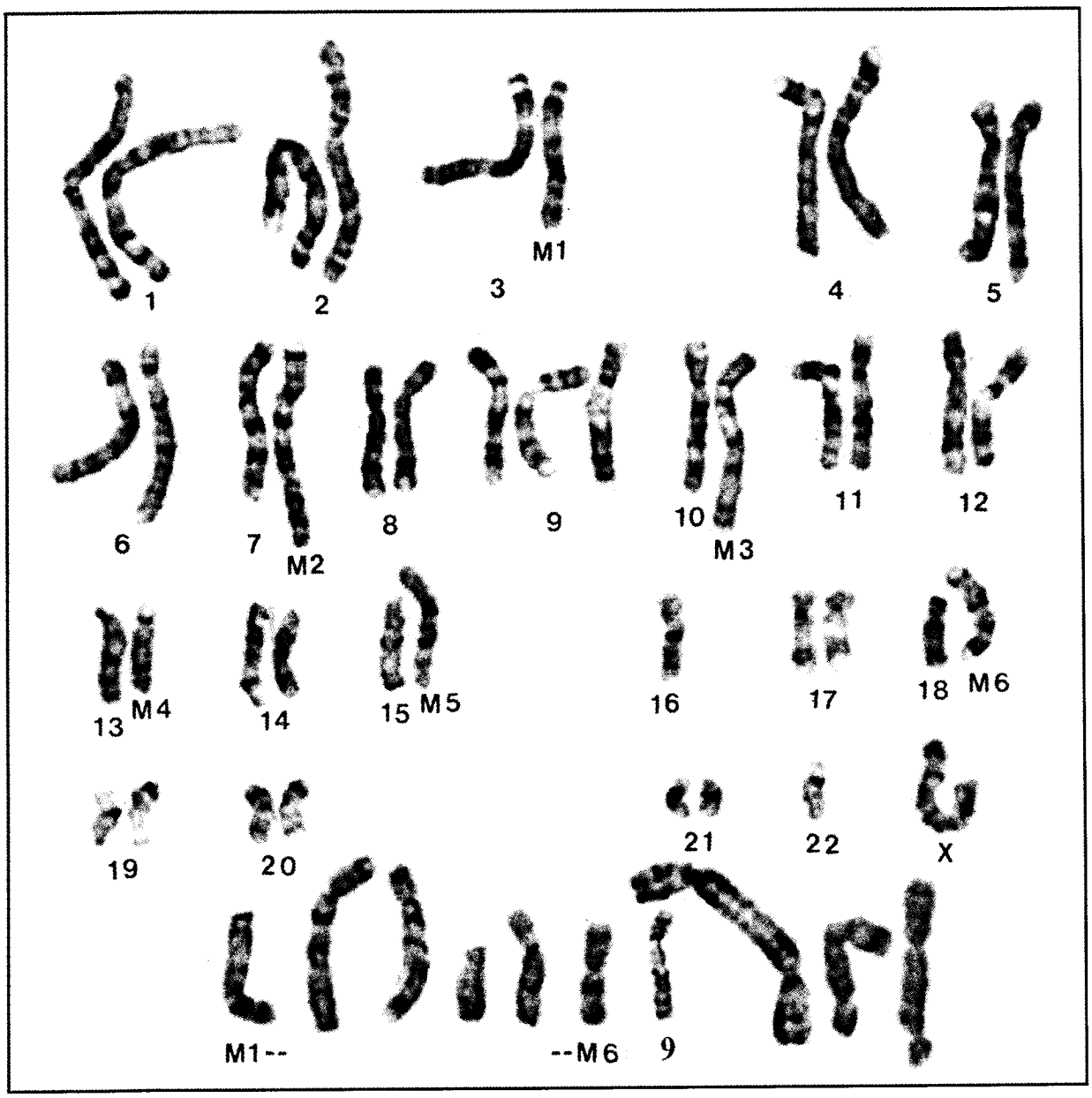
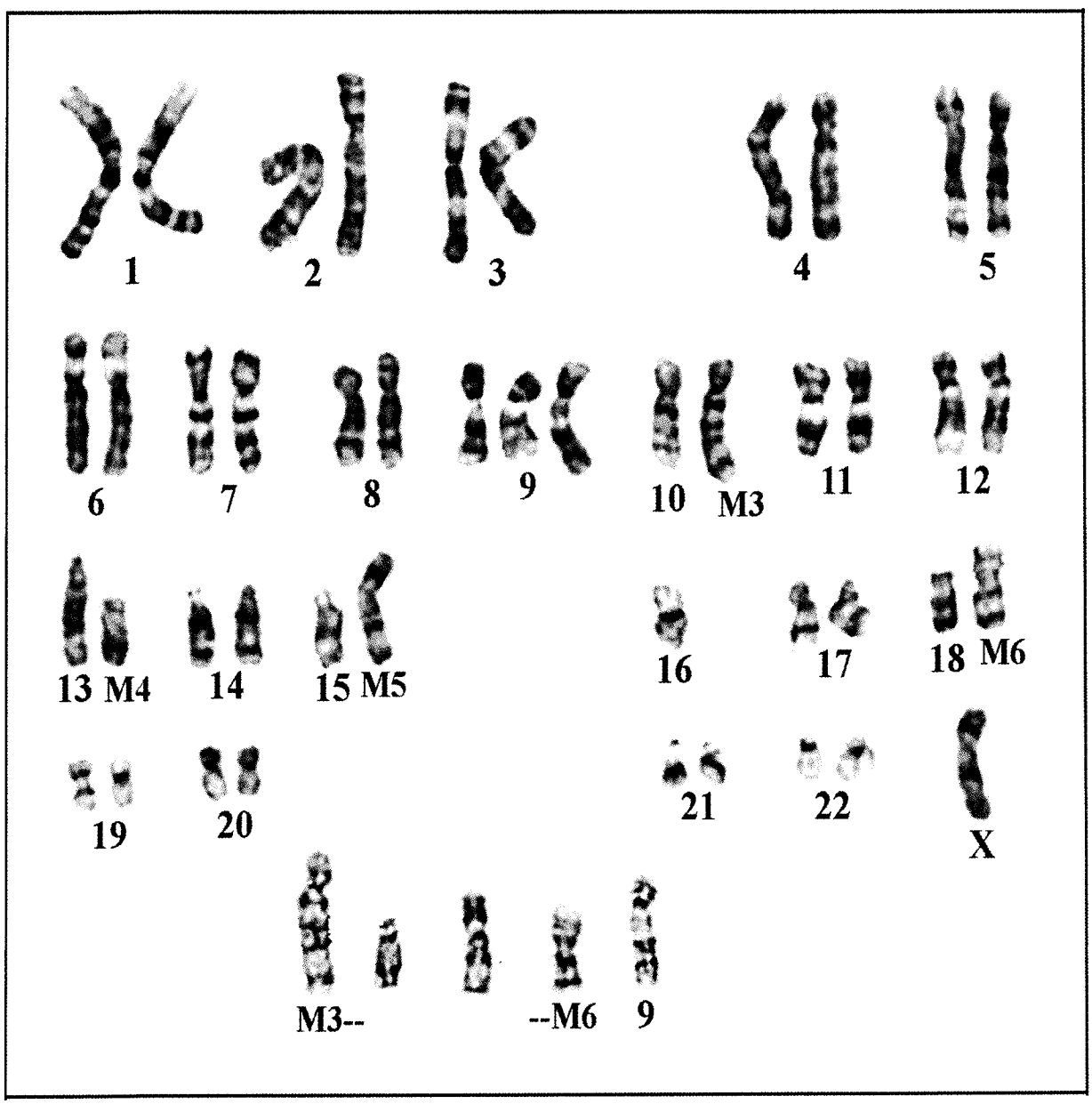

1
2
3
M1
4
5
6
7
M2
8
9
10
M3
11
12
13
M4
14
15
M5
16
17
18
M6
19
20
21
22
X
M1--
--M6
9

Figure 3c.



identified by G-banding (Figures 3a I. and 3b). These markers were tentatively identified as: M1=t (3p; 7q); M2=t (3q;7q); M3=t (10q;?); M4=del (13q); M5=t (15q;22q); M6=i (18q). At least four of these marker chromosomes, M3, M4, M5, M6, were also identified in HCT116-CloneK (Fig. 3c). The parental cell line showed two types of metaphases: one with all the markers and the other with only a few marker chromosomes. Furthermore, one of the three copies of chromosome 9 showed a large C-band region (lightly stained in G-banding) in the proximal q arm and this characteristic was present in cells from all three clones as well as the parental cells. Thus these studies confirmed that all three clones were of human origin and were derived from the same parental cell line HCT 116. Both clones 2 and K had significantly higher amounts of telomeric DNA as measured by Q-FISH (Fig. 4, Table 2). There was no apparent correlation between relative telomere area and the radiation sensitivity exhibited by these clones to treatment with fractionated or single dose X-radiation.

HCT116-CloneK, albeit the most radiation-sensitive, demonstrated the most resistant response to treatment with CPT, TPT, VP-16 or CDDP (Fig. 5). Specifically, in the case of CDDP, there was an inverse relation between response to X-radiation and to this particular drug (i.e. radioresistant HCT116-Clone2 was the most sensitive to CDDP; conversely, radiosensitive HCT116-CloneK was the most resistant to CDDP). The three clones, albeit having different relative responses to X-radiation and to the four drugs, had similar responses to UV-irradiation (Fig. 6).

Fig. 4 Q-FISH profiles of: (a) HCT116, (b) HCT116-Clone10, (c) HCT116-Clone2, and (d) HCT116-CloneK.

Figure 4.

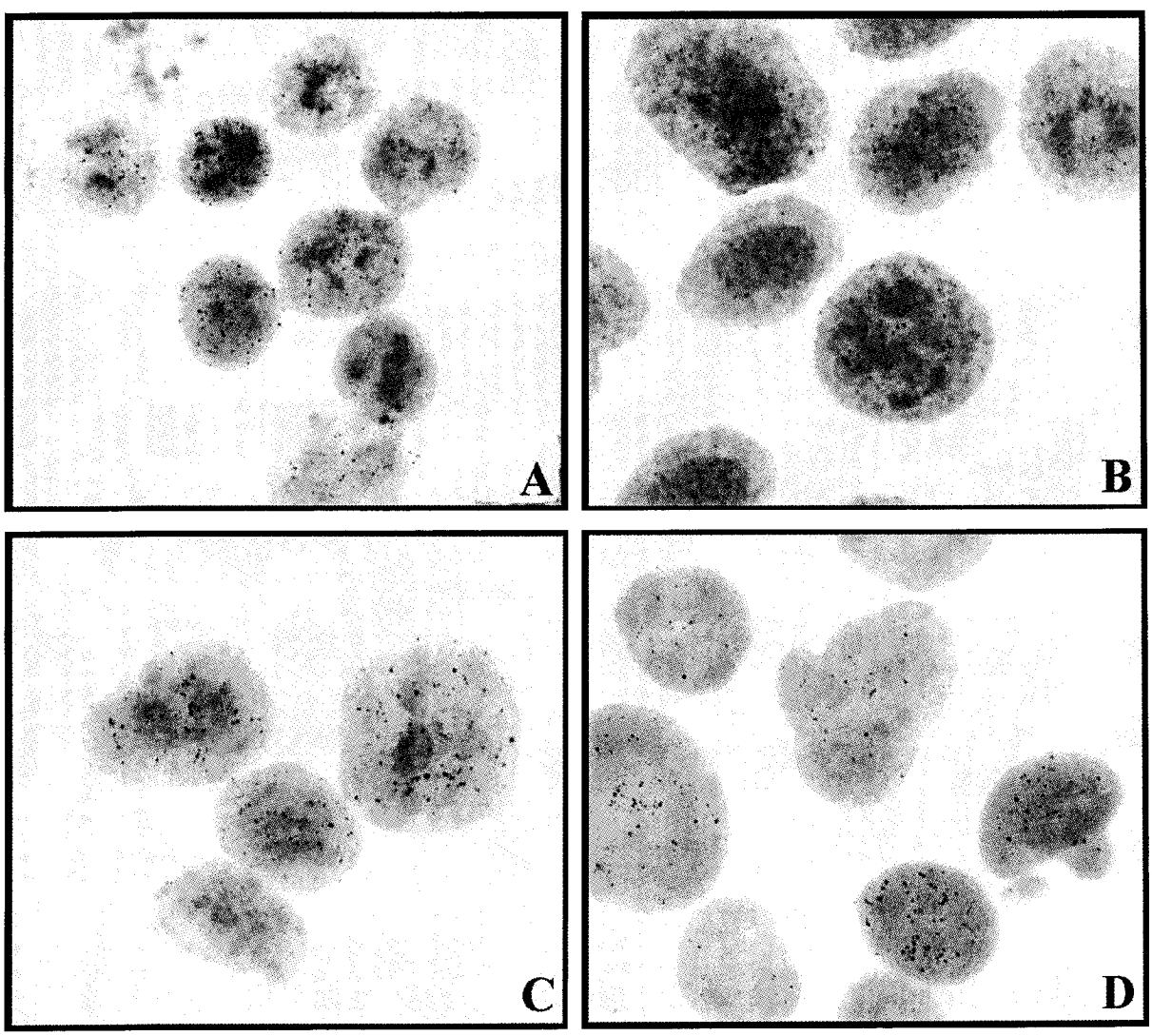


Table 2.

Cell line	Telomere area / Nuclear area, %	P value
Parental HCT116	0.760 ± 0.060	
Clone 10	0.648 ± 0.069	*HCT116 vs 10:* P < 0.10
Clone 2	1.443 ± 0.095	*HCT116 vs 2:* P < 0.01*
Clone K	1.702 ± 0.155	*HCT116 vs K:* P < 0.01*

All errors are the standard errors of the mean

Fig. 5 Clonogenic surviving fraction of the three clones following treatment (1 h exposure, 37 °C) with the four drugs. (a) CPT, (b) TPT, (c) VP-16, and (d) CDDP. Radiosensitive HCT116-CloneK expressed significant chemoresistance to CPT, TPT, VP-16, and CDDP relative to the other clones and parental cells. Each point represents the mean and S.E. of three independent experiments; within each experiment, each point was obtained by averaging 2-3 dishes.

Figure 5a.

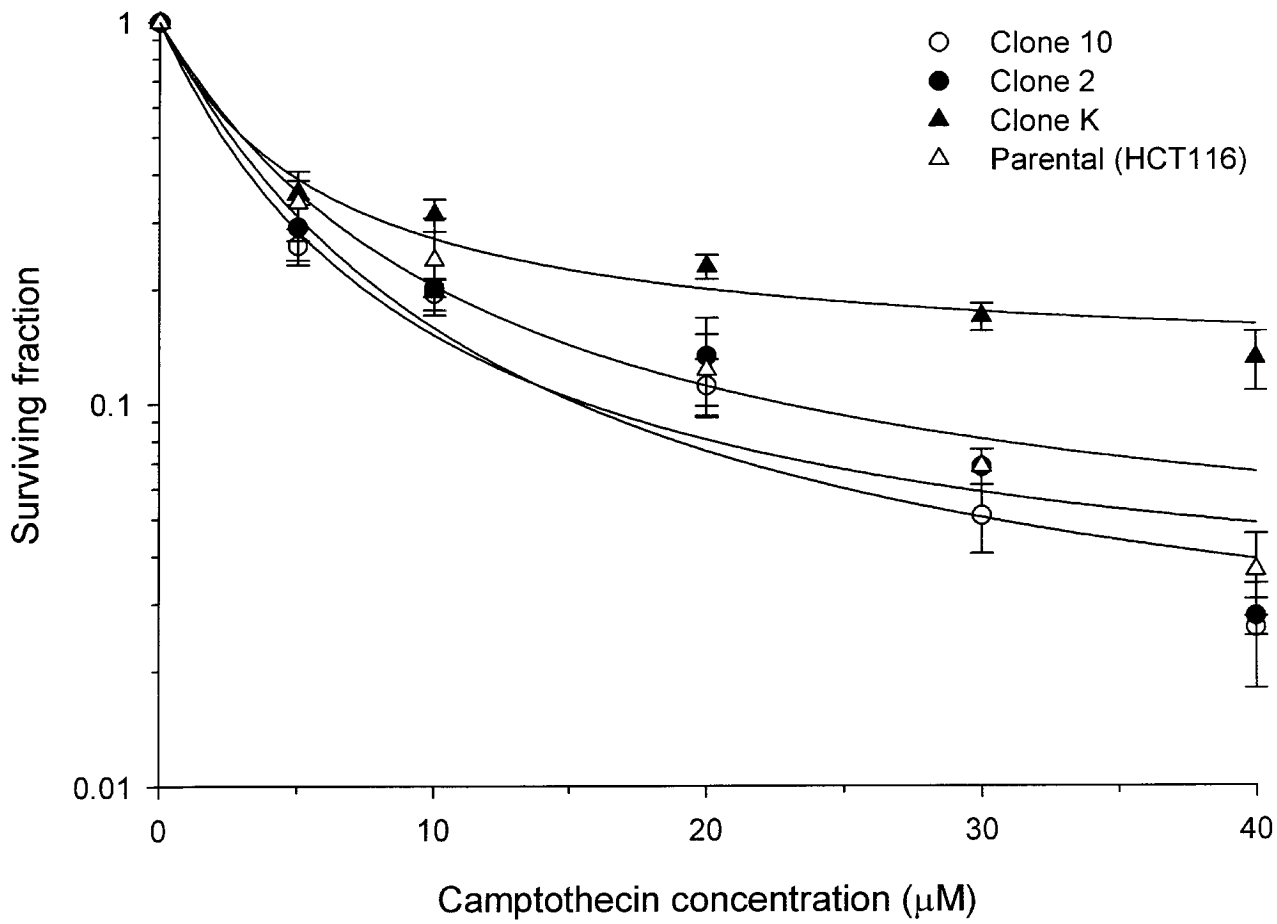

Clone 10
Clone 2
Clone K
Parental (HCT116)
Surviving fraction
1
0.1
0.01
0
10
20
30
40
Camptothecin concentration (μM)

Figure 5b.

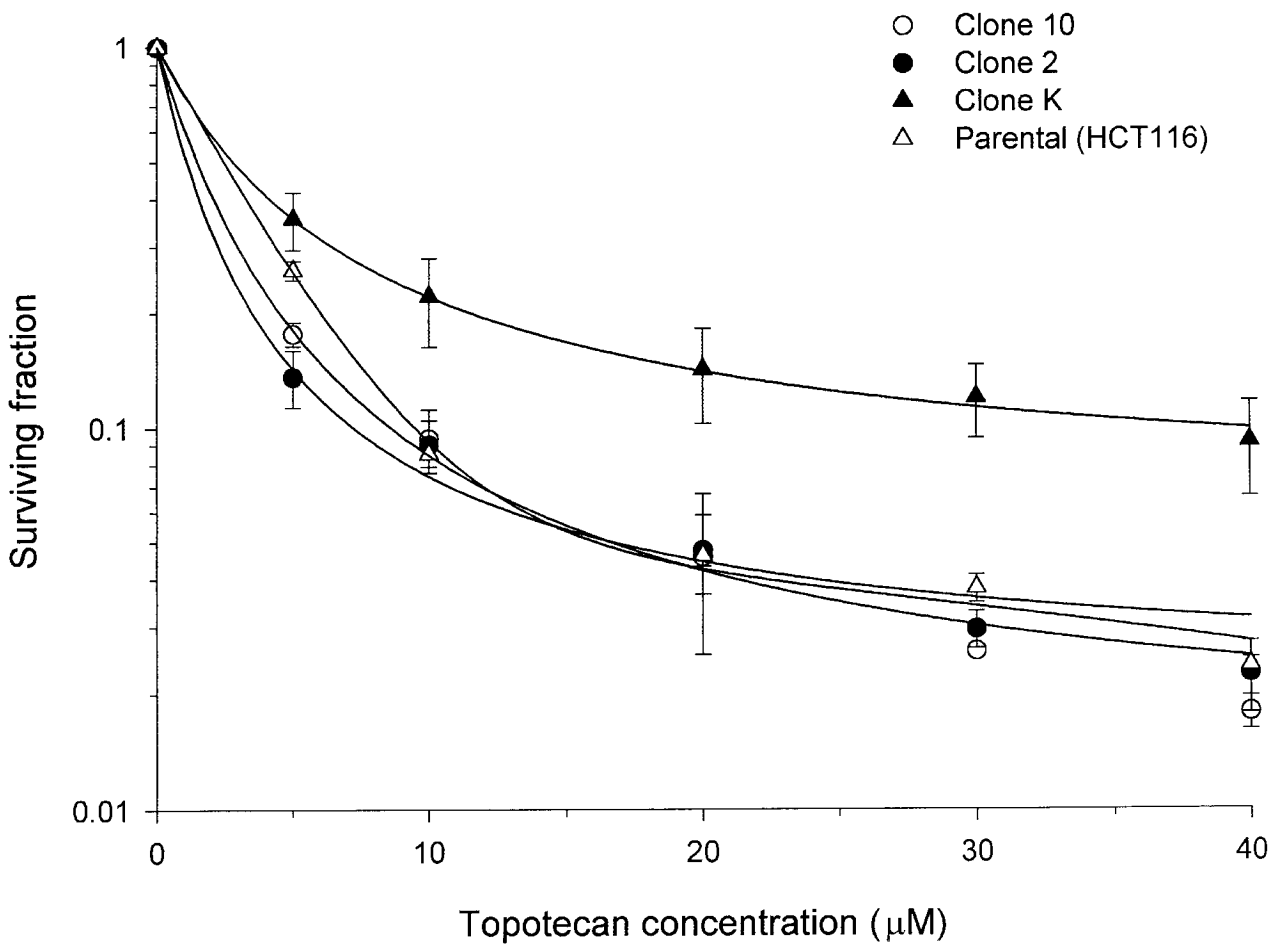

Clone 10
Clone 2
Clone K
Parental (HCT116)
Surviving fraction
1
0.1
0.01
0
10
20
30
40
Topotecan concentration (μM)

Figure 5c.

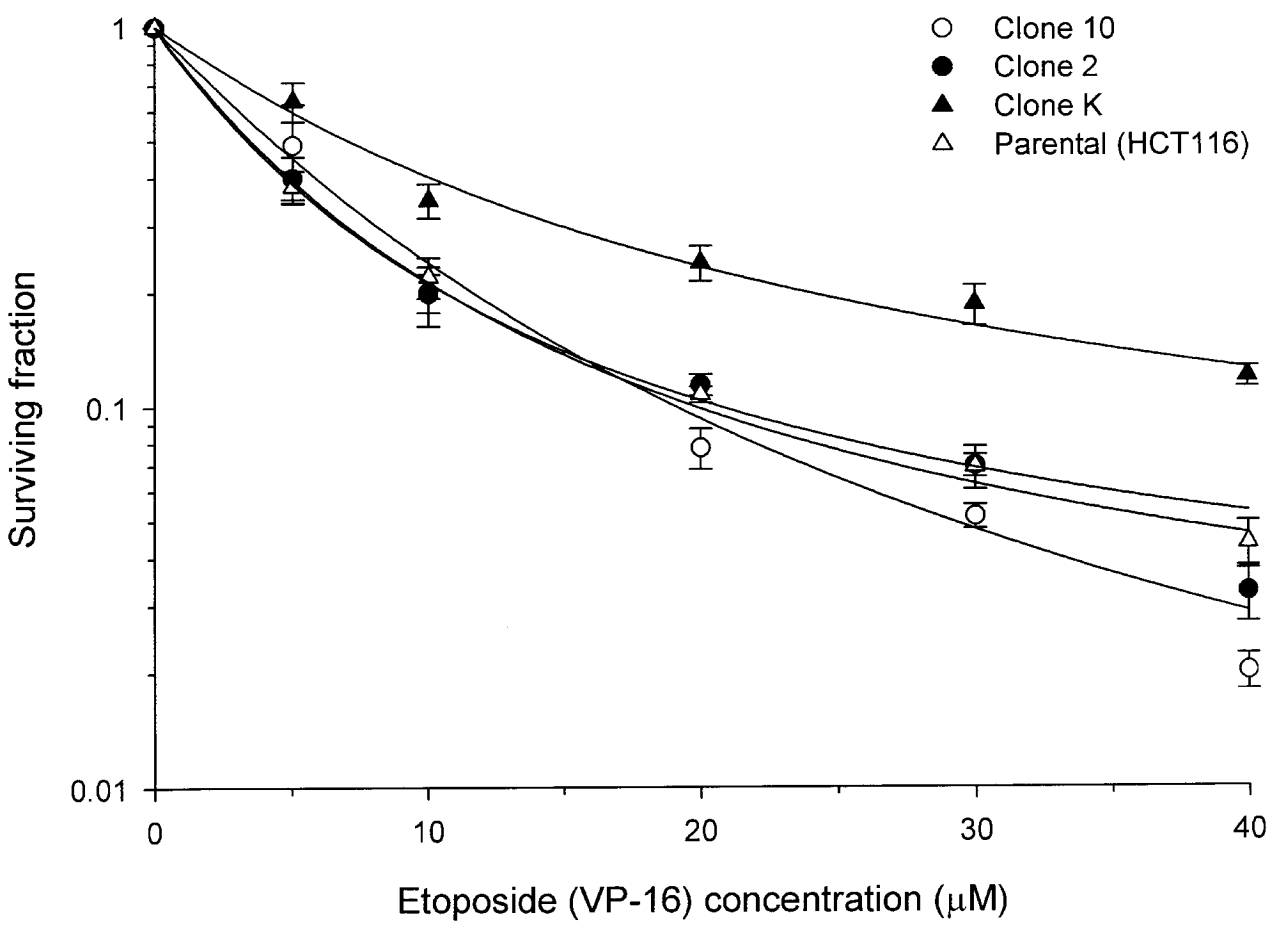

Clone 10
Clone 2
Clone K
Parental (HCT116)
Surviving fraction
1
0.1
0.01
0
10
20
30
40
Etoposide (VP-16) concentration (μM)

Figure 5d.

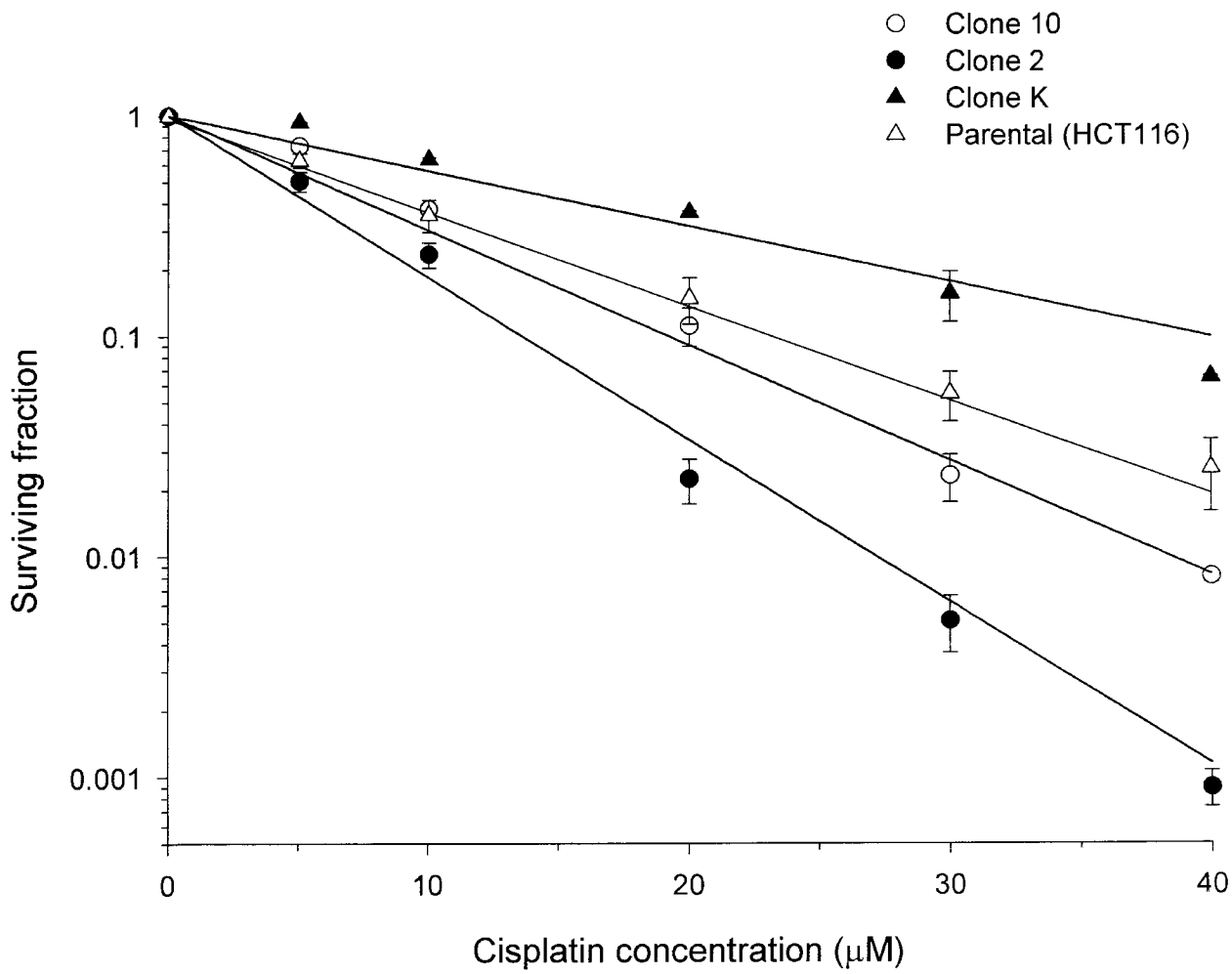
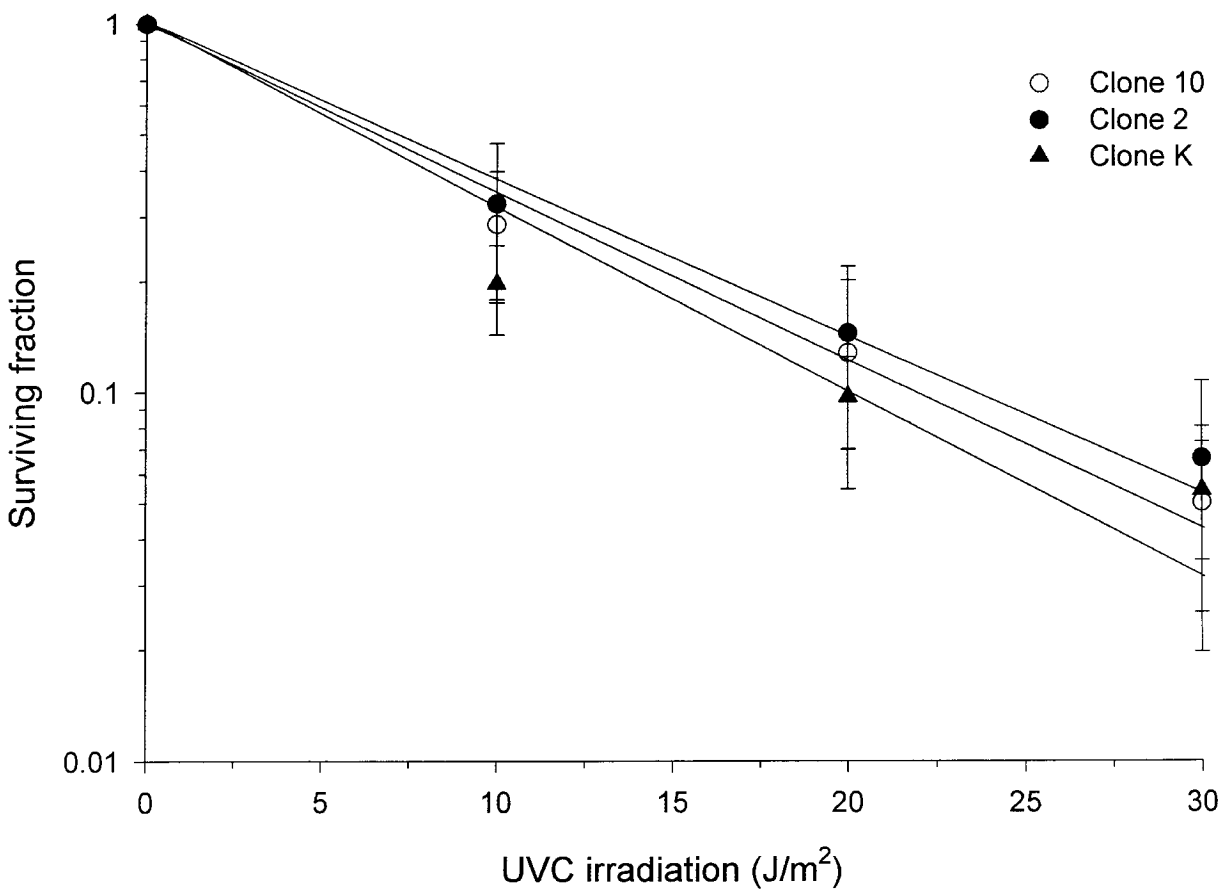

Clone 10
Clone 2
Clone K
Parental (HCT116)
Surviving fraction
1
0.1
0.01
0.001
0
10
20
30
40
Cisplatin concentration (μM)

Fig. 6 Clonogenic surviving fraction of the three clones following treatment with UV-radiation. Each point represents the mean and S.E. of two independent experiments; within each experiment, each point was obtained by averaging 2-3 dishes.

Figure 6.


1
0.1
0.01
Surviving fraction
0
5
10
15
20
25
30
UVC irradiation (J/m2)
Clone 10
Clone 2
Clone K

4.4 DISCUSSION

Many of the studies on the genetic basis of cellular response to X-radiation have benefited either from mutants that have increased sensitivity to ionizing radiation (IR) compared to the parental cell lines or from the study of human genetic syndromes that are associated with enhanced IR sensitivity (i.e. Fanconi' s anemia, Bloom's, Werner's) (17). By contrast, mutants or human genetic syndromes associated with increased IR resistance (as exemplified by Li Fraumeni) are rare. Thus it is interesting that one of our clones (HCT116-Clone2) is resistant to both fractionated and acute X-radiation.

All clones were isolated at the same time, expanded and frozen in liquid nitrogen until use. We attempted to keep the passage numbers approximately similar when the different clones were tested. It is worthwhile noting that even though there were individual differences among the clones, they were generally similar to each other and showed marker chromosomes that were present in parental cell line. Importantly, these data confirmed that: (a) the clones were of human origin and were derived from the same parental cell line, and (b) the untreated parental HCT 116 cell line was clonally heterogeneous with the presence of six marker (M1 to M6) chromosomes.

The heterogeneity of human tumours (i.e. existence of clones expressing different biological properties and/or response to treatment) has been of considerable interest (18-21). The presence of clonal heterogeneity in tumours has raised questions in regard to the biological origin and/or development of tumours (22-25). Clinically, clonal heterogeneity in

human tumours is often cited as a cause of treatment failure. This is because it is frequently assumed that a resistant subpopulation of cells (i.e. clone) survives and repopulates the tumour post-treatment (26, 27). There are many reports of sister clones/cell lines established either from primary tumours in vivo (18-21) or from previously treated cell populations (5-9) that express different biological characteristics and/or respond differently to treatment. The reasons for the presence of clonal heterogeneity are undoubtedly varied (e.g. due to selection pressures from the tumour microenvironment, uneven distribution of telomeric DNA in different chromosomes, treatment imparting genomic instability to the treated cell population). Conversely, there are relatively far fewer reports that have evaluated clonal heterogeneity in previously *untreated* cell lines. Among the few reported instances of clonal variation in X-radiation sensitivity from *untreated* cell populations are: (a) a human glioma cell line: SF_2 ranged from 0.33 to 0.61 (parental cell line SF_2 of 0.50) (10), (b) two clones of a human colon carcinoma cell line (DLD-1) with SF_2 of 0.53 and 0.8 (11), and (c) four clones of a lung carcinoma cell line (LX-1) with SF_2 values from 0.3 to 0.85 according to (11). Our present results have provided more evidence that clonal heterogeneity is a factor to be considered even in untreated cell populations in at least some cell lines.

HCT 116 cells were originally isolated from a male patient with colon carcinoma without any record of treatment (neither X-radiation nor drugs) (21). Three subpopulations (i.e. clones) with different morphologies were established from a single human colon carcinoma and named HCT 116, 116a and 116b. The latter two variant cell lines (HCT 116a, 116b) demonstrated morphological differences and also distinct histological patterns when grown as xenografts in mice (21). The ATCC obtained a culture of HCT 116 cells of

unknown passage in 1983 (28). ATCC described HCT 116 cells as epithelial-like with a near diploid stem line chromosome number (modal number of 45). According to ATCC also, some polyploid cells (6.8%) were present (28). We are not aware of any report in the literature that has compared the clonal variation in sensitivity to both fractionated (which is more relevant clinically) and acute X-radiation of HCT 116 cells. Because fractionated treatment involved factors that were absent during acute treatment (e.g. repair, cell cycle redistribution), it is interesting that we have found clones that were either resistant (HCT116-Clone2) or sensitive (HCT116-CloneK) to *both* these types of treatment. One possible explanation is that there is a genetic basis for the response of these two clones to X-radiation. In fact, using DNA microarrays, we have identified genes that are differentially expressed in these clones (29).

Telomeres are associated with the maintenance of chromosomal stability (30). In yeast, telomeres affect cellular response to ionizing radiation and DNA double strand break repair proteins, including Ku, are present in yeast telomeres (30). In mammalian cells, telomere length was reduced seven-fold in radiosensitive cells relative to radioresistant cells (31). The latter study suggested that there might be a possible linkage between abnormal telomere length dynamics and the radiosensitive phenotype. We, therefore, wondered whether there was such a linkage in our sister HCT 116 clones. Our results, however, did not show that there was an apparent correlation between relative telomeric DNA content of our clones and the X-radiation response since both clones 2 (radioresistant) and K (radiosensitive) had significantly increased telomere content compared to the control HCT116-Clone10.

HCT116-CloneK, which was relatively the most sensitive to X-radiation, was the most resistant to the four chemotherapeutic drugs (CPT, TPT, VP-16 and CDDP). There were no significant differences, however, in the response of these three clones (2, 10, K) to UV-radiation. The drugs in this study had been chosen for their relevance to chemotherapy/chemoradiation and for their diverse mechanisms of action (32). CPT and TPT are inhibitors of DNA topoisomerase I (32), VP-16 is an inhibitor of DNA topoisomerase II (32) and CDDP forms intra- and inter-strand DNA adducts (14). It is well known that cellular resistance to X-radiation is frequently directly correlated to resistance to CDDP (33-36). Additionally, there are reports of cross-resistance between CDDP and UV-radiation (37). Moreover, cells that have been exposed to fractionated X-radiation treatment can also become resistant to CDDP (38).

Although it has been suggested that glutathione (39) or Ku-antigen (37) may play a role, the actual molecular mechanisms that are responsible for the cross-resistance between X-radiation and CDDP have not yet been established. On the other hand, it is now known that genetic factors are also important in determining cellular response to drug treatment. For example, genes involved in the apoptotic pathways (40) have been implicated in the response of many cell types to different drugs. Thus, assuming that there is a genetic basis for the response of our clones to the drug treatments given, our results suggested that at least some of the genetic pathways that modulate response to X-radiation and response to these drugs were non-overlapping in HCT116-CloneK. HCT116-CloneK in particular may provide the opportunity to differentiate some of the genetic pathways that modulate X-radiation response from those that modulate chemotherapeutic response. This would not have been possible

with cell lines/clones that show cross-resistance to both X-radiation and drugs. Our results also suggested that at least some of the genetic pathways that influence the responses of these clones to X-radiation, drug and UV-radiation do not overlap. Therefore, we propose that these clones may be useful models for preclinical studies determining the genetic basis of the response of human colorectal cancer cells (and perhaps for tumour cells generally) to these treatment agents.

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Qutob, S. S., Mesak, F. M, .Proulx, D., Liu, Q. Y., Walker, P. R., and Ng, C. E., Genome-Wide Analysis of Gene Expression in Unirradiated HCT116 Cell Clones Exhibiting X-Radiation Resistant and Sensitive Responses Reveals a Possible Role for Spermidine/Spermine *N*1-Acetyltransferase (*SSAT*). *Radiat. Res.*

5. ABSTRACT

We had previously isolated clones with a wide range of X-radiation (XR) responses from human colorectal cancer HCT116 cells. These included cell clones that were resistant (HCT116-Clone2), sensitive (HCT116-CloneK) or similar in response (HCT116-Clone10) to both fractionated and acute XR as the parental cells. To determine the basal differences in gene expression among these unirradiated cell clones, we performed genome-wide analysis of gene expression using cDNA microarray that contained 19,200 ESTs. We found 2 or 5 genes, respectively, that were differentially expressed in HCT116-Clone2 or HCT116-CloneK relative to HCT116-Clone10 cells. Following verification with quantitative (Q)-PCR, only four genes (*APM2*, *XRRA1*, *SSAT*, and *CYP24A1*) were identified to have more than 2-fold changes. Of these four genes, Spermidine/Spermine N^1-Acetyltransferase (*SSAT*), an enzyme that catabolizes polyamines which are known to protect cells from XR damage, was the only gene with a known potential link to XR response. *SSAT* was upregulated (2.3-fold by microarray or 6.3-fold by Q-PCR) in HCT116-CloneK. *SSAT* protein levels and its enzymatic activity in the presence of DENSPM, a known inducer of *SSAT*, were relatively the highest in the unirradiated radiosensitive HCT116-CloneK. Interestingly, treatment with XR and DENSPM was associated with decreased survival and significant sensitizer enhancement

ratio (SER) values in all cell clones except in HCT116-CloneK when compared to XR alone. Intracellular polyamine levels were also found to be correlated with the (SER) values of the clones and the radioresponse of HCT116-Clone2. Furthermore, XR treatment, in the presence of DENSPM, was discovered to decrease intracellular polyamine levels but various doses of XR was unable to significantly increase DENSPM-stimulated *SSAT* activity in all three cell clones suggesting that that the radiation response exhibited by the cell clones may be a result of a number of mechanisms including those that regulate polyamine levels. Taken together, our findings suggest that genome-wide profiling of gene expression in unirradiated tumour cells may be useful for identifying genes that can affect their XR responses.

5.1 INTRODUCTION

Radiotherapy and chemoradiation are major modalities currently employed for treating cancer. Understanding the basis of tumour and normal cellular response to X-radiation (XR) may permit us to improve treatment efficacy and minimize undesired normal tissue damage. Much evidence supports a genetic basis for the manifestation of either resistance or sensitivity to XR. This evidence includes the finding that genes or genetic pathways involved with cell cycle control, DNA repair and/or cell death are commonly invoked in response to genotoxic stress, including that caused by XR (1). However, because of the complexity of these genetic pathways, much remains to be learned about the molecular basis of cellular response to genotoxic stresses.

DNA microarray technology has made it possible to screen gene expression rapidly and on a genome-wide basis (2). However, we are aware of only a few published studies that have used microarrays to identify the genes that are potentially involved in XR resistance or sensitivity (3-9). Recently, two reports applied gene expression profiling analysis of yeasts carrying deletions in 3670 or 4627 nonessential genes to identify genes that conferred either resistance to γ-radiation (10) or sensitivity to UV-radiation (11). However, in subsequent experiments, Birrell et al. (12) were unable to identify the genes that conferred protection against various DNA damaging agents (ionizing radiation, UV, cisplatin and H_2O_2). In general, all the published studies have evaluated differences in gene expression between XR-resistant versus XR-sensitive established human cell lines, cells derived from primary tumours or in yeast cells (13) following exposure to XR. These studies have not examined whether global gene expression profiling of *unirradiated* clones derived from a parental cell line can identify genes that are important when these cells are subsequently challenged with XR.

We had previously isolated clones with a wide range of XR responses from human colorectal cancer HCT116 cells (14). These included only one cell clone that was resistant to both fractionated and acute XR treatment (HCT116-Clone2), a cell clone that was sensitive to both types of XR treatments (HCT116-CloneK), and cell clones (i.e. HCT116-Clone10) that had similar responses to both types of treatment as the parental HCT116 cells. To determine the basal differences in gene expression among these unirradiated cell clones, we performed genome-wide analysis of gene expression using cDNA microarrays. Although we identified several genes that were differentially expressed in these clones, we could only find one gene, Spermidine/Spermine N^1-Acetyltransferase (*SSAT*), that had a known potential link to XR response.

SSAT is the rate-limiting enzyme in polyamine catabolism. Polyamines (putrescine, spermidine and spermine) are aliphatic cations synthesized from the amino acid precursor L-arginine and L-methionine by the enzymes ornithine decarboxylase and S-adenosylmethionine decarboxylase (15). Polyamines are associated with the cell cycle, cell division, tissue growth, and differentiation (16). Interestingly, polyamines have also been found to be a potent radioprotector of DNA and that polyamine-depleted cells become radiosensitive (17). Polyamines, particularly spermine, confers radioresistance by binding to the minor groove of the DNA strand, triggering a reduction of the accessibility of radiolytic attack sites to OH· radicals (18). Treatment with the spermine analog N^1, N^{11}-diethylnorspermine (DENSPM) can efficiently deplete cellular pools of polyamines by significantly upregulating the activity of *SSAT* and also downregulating polyamine biosynthetic enzymes (19). Depletion of cellular polyamines can inhibit DNA double strand break (DSB) repair (20, 21). This inhibition of repair may be due to a modulation of the DNA-protein interaction necessary for the non-homologous end joining of DNA

double strand breaks. Additionally, depletion of cellular polyamines can also activate a cascade of molecular signals leading to cell cycle arrest or cell death (22). *SSAT* converts spermine or spermidine to the acetylated form: acetyl-spermine or acetyl-spermidine (23). Acetylation of polyamines facilitates their degradation or export out of the cell, presumably to regulate cytotoxicity arising from increased intracellular polyamine content. It was not until recently that the Ichimura group (24) claimed to have direct evidence that XR-induced accumulation of *SSAT* mRNA in HeLa S3 cells might be due to activation of the polyamine-responsive element (PRE). Although Amundson SA et al. (9) detected increasing levels of *SSAT* in ML-1 cells employing a 1238 ESTs (cDNA) microarray, it has not been established that elevation of *SSAT* mRNA level correlates with its cellular activity and predisposes cells to killing by XR. Our report here further examines the role of *SSAT* gene expression in determining XR response in HCT116 clones. Polyamine and *SSAT* levels as well as *SSAT* enzymatic activity in HCT116 cell clones in the presence of DENSPM and in combined modality with XR are also discussed.

5.2 MATERIALS AND METHODS

5.2.1 *Cloning of HCT116-Clone2, HCT116-CloneK and HCT116-Clone10 Cells:* Human colorectal tumour (HCT116) cells were a kind gift from Dr. Vogelstein of The Johns Hopkins University School of Medicine. We cultured the cells in DMEM/Ham's F12 1:1 mix (Wisent Inc., St. Bruno, QC, Canada) supplemented with 10% fetal bovine serum (Wisent Inc.) and 15 mM HEPES buffer in a humidified atmosphere of 95 % air, 5 % CO_2 at 37°C. HCT116 cells were trypsinized and serially diluted in complete medium in 24-well, flat bottom Falcon MULTIWELL ^{tm} tissue culture plates (Becton Dickinson,

Lincoln Park, NJ, USA). Wells with one single colony were identified by light microscopy and subsequently marked. Cells of the marked colonies were recovered and were subsequently plated in one well of a 6-well plate and grown to 70% confluence. The cells were then seeded into a 25-cm^2 flask for two weeks. Sub culturing into 75-cm^2 flasks was repeated. The HCT116 cell clones denoted as HCT116-Clone2, HCT116-CloneK, and HCT116-Clone10 used for this study were chosen for their XR resistance, sensitivity, and similarity to parental HCT116, respectively (14).

5.2.2 *cDNA Microarray analysis of HCT116-Clone2 versus HCT116-Clone10 and HCT116-CloneK versus HCT116-Clone10:* Total RNA was extracted using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen Inc, Mississauga, ON, Canada). Fluorescently-labelled first strand cDNAs were hybridized to the Human 19K microarray slides obtained from The Microarray Center of the University Health Network, Toronto, Canada (http://www.microarrays.ca/). The two slide set contained 19,200 characterized and unknown human ESTs together with a number of control features. The slides were designated Slide A and Slide B. Each slide had 9,600 ESTs spotted in duplicate and was organized into 24 sub-arrays of 600 spots each. The hybridizations were carried out using HCT116-Clone10 as a control sample (usually labeled with Cy3) and the HCT116-clone2 or HCT116-cloneK as experimental samples (labeled with Cy5) in a competitive hybridization reaction. One reciprocal labeling was performed for each sample and at least three replicate hybridizations were performed for each experiment.

Each fluorescently-labelled cDNA was generated in 40 µl of reaction mix containing 1X superscript II first strand buffer (Invitrogen), 3.75 pmoles/µl AncT mRNA

primer (5' T_{20}VN 3'), 0.5 nmoles/μl each dATP, dGTP and dTTP, 50 pmoles/μl dCTP, 25 pmoles/μl Cy3- or Cy5-conjugated dCTP (all Amersham Biosciences, Baie d'Urfe, QC), 10 nmoles/μl DTT (Invitrogen) and 20 μg total RNA. The reaction mixture was first heated to 65°C for 5 min and then cooled to 42°C for another 5 min to denature the RNA and anneal the AncT primer. Reverse transcription was accomplished by adding 2 μl of Superscript II reverse transcriptase (Invitrogen Life Technologies, Burlington, ON) and 1 μl of RNase inhibitor (Promega, Madison, WI) and incubation at 42°C for 2-3 h. The reaction was stopped by adding 5 μl of 50 mM EDTA and the RNA templates were hydrolyzed by adding 2 μl of 10 N NaOH to the cDNA reaction, followed by an incubation at 65°C for 20 min. The reaction was then neutralized by adding 4 μl of 5 M acetic acid.

Before hybridization, the Cy3 and Cy5 probes were combined and precipitated with an equal volume of isopropanol. The pellet was washed with 70% ethanol and air-dried in the dark. The labeled cDNA probe mix was then resuspened in 5μl water and combined with 80μl of DIG Easy Hyb buffer (Boehringer Manheim, Germany) containing 0.5μg/μl yeast tRNA and salmon sperm DNA. This hybridization solution was heated to 65°C for 2 min, cooled to room temperature and injected between a paired set of the human 19K microarray slides. Hybridization was carried out in the dark at 37°C for 18 h. After hybridization, the slides were washed three times in 1x SSC containing 10% SDS for 10 min, plus a final wash with 1x SSC alone. The slides were dried by centrifugation at 40xg in a Sigma 4K 15 centrifuge for 5 min.

5.2.3 *Microarray data acquisition:* The slides were scanned using a ScanArray 5000 confocal scanner (Packard BioScience, Meriden CT, USA) with excitation/emission wavelengths of 543 ηm / 570 ηm for Cy3 and 633 ηm / 670 ηm for Cy5, at 10μm resolution. Hybridized slides are scanned first in the Cy5 channel and then the Cy3 channel, as Cy5 is more susceptible to photodegradation than Cy3. The resulting 16-bit grayscale image files, one for each channel, were quantified together with QuantArray v3.0 software (Packard BioScience) using an adaptive spot finding method to generate spot intensities from mean pixel values. Local area background measurements were derived from a background mask (doughnut) surrounding the spot. Poor quality spots were flagged manually by the user and recorded in the output file to be used as an "ignore spot" filter. The tab delimited text data files produced were subsequently pre-processed using macros in Microsoft Excel 2000 (Microsoft Corporation, Redmond, WA).

5.2.4 *Microarray data pre-processing:* Each data file contained intensity data for 19200 features (i.e. 9,600 ESTs in duplicate) measured in two channels, Ch1 and Ch2. Ch1 data represented intensity measurements from the control sample and Ch2 data represented intensity measurements from the experimental sample. Median subarray background values were calculated for each channel and subtracted from the respective intensity values. Spots flagged by the user during quantification (the "ignore" filter) and spots failing to meet the following criteria; intensity > 2.5-fold background and intensity > 5^{th}- and < 98^{th}-percentile of all intensities for each channel, were filtered out and not used in the computation of normalisation correction factors. Corrected intensity data were logged (base 2) and corrected for dye bias (normalised) using a linear-regression correction applied to the Ch2 intensities for all the spots in each subarray. This correction

yielded a Ch2 versus Ch1 scatter plot with a linear regression best-fit line having slope 1 and intercept 0. Log_2 ratios representing expression values for experimental versus control samples were then calculated and data was analysed in Excel to select for spots with a mean log ratio of at least 0.8 for which all three replicates were present.

5.2.5 *Q-PCR analysis:* First strand cDNA was generated in a total volume of 40 µl per 20 µg RNA aliquot, in a reaction mix containing 0.5 nmoles/µl dNTP (Invitrogen) instead of the nucleotide mixture for microarray. Reverse transcription was carried out for 2 hours at 42 °C, reaction stopped by adding 5 µl of 500 mM EDTA, RNA hydrolysed and the mix neutralised as described. cDNA was purified and quantified using a Qiaquick PCR kit (Qiagen) and Oligreen dye (Molecular Probes), following the manufacturer's instructions. For Q-PCR, 1.2-2 ng cDNA was amplified in 25 µl of SYBRgreen PCR master mix (Qiagen, CA, USA) containing 0.15 pM each of gene-specific forward and reverse primers. Fluorescent product was detected using an ABI Prism 5700 system, set for 1 cycle of 2 minutes at 50 °C, 1 cycle of 10 minutes at 90 °C and 40-43 cycles of 15 seconds at 90 °C, 1 minute at 60 °C. Product dissociation was also monitored to confirm specificity of the primers. Forward and reverse primers for sequences of interest were designed using Primer Express (Applied Biosystems) and are listed in Table 1.

Table 1. List of oligos used in Q-PCR verification of cDNA microarray. These oligos were derived from ESTs that were spotted onto the glass microarray (University Health Network, Toronto) and the primer design program, Primer Express v2.0 software (Applied Biosystems, Foster City, CA, USA) was use to design functional primers of 20 nucleotides in length of a guanine (G) or cytosine (C) content of no more than 20 to 80% containing no more than two G's or C's within the five nucleotides at the 5' or 3' ends of the primer.

Table 1.

Q-PCR oligos					
No.	*Gene name*	*Accession No. (EST)*	*Gene id.*	*Forward*	*Reverse*
1	TCL6;TNG1;TNG2	T96687	121404	AGACAGGCTGGAGTTGCTGAA	TGAGTCCAATTCCACCCTGATT
2	HLA-B	H63653	207215	CGAGGACCAAACTCAGGACACT	CCACAGCTGCCCACTTCTG
3	Unknown EST	AA131311	503573	GAGTTCTGCCACCCTGAACATT	GTCAGACCACTCCCACAAAGG
4	MCP1; SCYA2	AA047099	488534	AGCAGAAGTGGGTTCAGGATTC	AGTGAGTGTTCAAGTCTTCGGAGTT
5	XRRA1	R40588	27795	CTGACAGAAACATCCCCAACAG	CCTCCTCTCCAACTCATCAGAAG
6	Unknown EST	R26736	133298	GCAATCCATTTCGGCAAAGA	GCACTGGCCACTCCAGTCA
7	Unknown EST	T89477	116799	ACAAAGCTATTTTCCTCGAACACA	ACCCCCAACCCCTAGCAA
8	APM2	H43908	183315	CAAGACCACCCAGGAAACCAT	TTTTTTCCCGATCCCAGAGA
9	SSAT	H03112	152226	CGGGCCGACTGGTGTTTA	GGAGCGTCCTCTTCTCAGTCA
10	GSK3A	T72585	22047	TGAGGGAACTTGAACTCCGTGTA	TCATCAAGGTGCTGGGAACA
11	NFATC3	N92360	308119	AAGCAAAGTTCATTTCCCCAAA	CACCTCAGGCCTTGGGTAGA
12	Unknown EST	AA195088	665280	AACAGGCATTACTGGGAATCC	CAGGACCTCACATTGAGGTAAGG
13	S100 A3	AA055241	377441	GGACTTGCCCAGGTCTTCTG	CTGAGGAAGGAGCCCTCACAT
14	ADH6	AA040747	486189	TCCAAGTGGCCTGAGTATTTCA	GAAATTGCACATCATCATTTGGAT
15	MARK2	H19443	51515	CGGATCCCTGCCTTTTGA	GGAATACGGTATTTTCCCCTCAGTA
16	TAB1; TAK1-binding protein	R40486	27920	TGCAGGTGAGAGAGGATTTAAAGTCA	CAGCACCACAGAGGCATTCTT
17	CYP24A1	H96028	250027	TTGGCGTTGGAAAAAGAATGT	ACAATCCAACAAAGAGCCAAATG

5.2.6 *X-Radiation of HCT116 Cell Clones:* $2x10^5$ cells were seeded into a 25 cm^2 flask on day 0 and used for experimentation on day 3 such that at the start of each treatment cells were in the exponential-phase of growth. These were then treated with either 4 or 8 Gy of acute (i.e.single) doses of XR at room temperature. A 250 kVp X-ray unit (Pantak, CT, USA) was used at a dose-rate of 150 cGy/min.

N^1,N^{13}-Diethylnorspermine (DENSPM) was purchased from Tocris, Ellisville, MO, USA and dissolved in sterile water. Stock solutions of 10 mM were prepared and the appropriate amount was added to the culture medium of the cells. All DENSPM exposures were for 24 h at 37 °C. For combined DENSPM and XR treatments, XR was given before DENSPM with the exception of the clonogenic assay experiments where XR was given after treatment with DENSPM.

5.2.7 *Clonogenic assay:* The cells were rinsed twice with isotonic citrate saline solution immediately following treatment with acute X-radiation or immediately after the 24 h treatment period for the experiments involving X-radiation and DENSPM. In either case, the cells were then trypsinized (0.2% trypsin/2.5 mM EDTA for 5 min at 37 °C) and counted with an electronic particle counter (Particle Data Inc., Elmhurst, IL). We plated cell suspensions to yield about 50 colonies per 60 mm dish after 14 days in a humidified atmosphere of air containing 5 % CO_2. Three dishes were plated for each dose in every experiment and we carried out three independent experiments for each clone for each treatment. We stained dishes with methylene blue and colonies containing more than 50 cells were scored to assess surviving fraction. We fitted radiation survival curves with the linear quadratic model $S = \exp(-\alpha D - \beta D^2)$. Sensitizer enhancement ratios (SER) were

calculated from the ratio of survivals following XR alone to those following combined XR and DENSPM treatment for the respective clone (Table 3).

5.2.8 *SSAT Activity Assay:* Exponentially-growing cells were plated in triplicate at $3x10^4/cm^2$ in 96-well plates. Cells were incubated for 24 hours with or without 20 μM DENSPM. Cells were then exposed to single doses of XR at 4 or 8 Gy 24 hours prior to the assay. The SSAT activity was then assayed by measuring the conversion of [1-^{14}C]acetyl-CoA into [1-^{14}C]acetylspermidine as described by Matsui *et al., 1981* (25). Briefly, following the 24 hour treatment period, the culture medium was removed and replaced with the assay reagents containing 50 mM Tris-HCl (pH 7.8), 3 mM spermidine (N-[3-Aminopropyl]-1,4-butanediamine) (Sigma, St. Louis, MO, USA), and 16 μM (55 mCi/mmol) [1-^{14}C]acetyl-CoA (ICN Biochemicals, Costa Mesa, CA, USA) in a total volume of 100 μl. The enzymatic assay solution was then added to the cells at 30°C for 15 min. The reaction was terminated by adding 20 μl of 1 M hydroxylamine hydrochloride ($NH_2OH•HCl$) and heated in a boiling water bath for 3 minutes. 100 μl of the reaction mixture were added to a cellulose phosphate paper disc (2.3 cm; Whatman p81), which was then rinsed 5 times in distilled water and 3 times with ethanol on 2.3 cm Whatman sintered glass microfibre filter. The paper p81 disc was dried and the amount of radioactive acetylspermidine was measured using a Packard 1900TR liquid scintillation analyzer with the addition of 10 ml of CytoScint counting fluid (ICN Biomedicals, Irvine, CA, USA).

5.2.9 *Western Blot Analysis:* Cells were lysed by sonication for 30 sec at 4°C in Frack's lysate buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5 mM EDTA, and 20% glycerol with 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 200 μM NaF, and 200 μM sodium PPi). Proteins present in the cell lysates were resolved by SDS-polyacrylamide gel electrophoresis using a 10% gel (100 μg / lane) and electrotransfered to polyvinylidene difluoride membrane Hybond-P (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Hybridization with rabbit polyclonal anti-*SSAT* antibody (generously provided from Dr. Anthony Pegg, Pennstate College of Medicine, Hershey, PA, USA) was followed by immunodetection with ECL-Plus Western blot detection kit (Amersham Pharmacia Biotech). Band intensities were quantified using the Area Density Tool algorithm of the LabWorks software program analyzing images obtained with an Epi Chem II Darkroom instrument (UVP Inc.).

5.2.10 *Determination of Polyamine Content in HCT116 Cell Clones with HPLC:* Intracellular contents of polyamines were measured using high performance liquid chromatography (HPLC) with a spectrofluorometer (wavelength e.g. 342 nm excitation and 512 nm emission). Exponentially growing cells at 70% confluency were typsinized and harvested in 500 μL of ddH_2O. Cells were lysed by sonication for 10 sec at 4°C and 25 μL was taken out for Bio-Rad DC Protein Assay quantification (Bio-Rad Laboratories, Hercules, CA, USA). Ten percent perchloric acid was added to the remaining lysate solution for protein degradation. This mixture was centrifuged at 3000 g for 10 minutes and the supernatant collected. The supernatant was added to saturated sodium chloride, 0.4 mM diaminoheptane internal standard and 1 ml of dansyl chloride (5 g/L in acetone). The dansylation reaction was conducted on a heating block at 60°C for one hour. Following this

procedure, 2 ml of cyclohexane was added to the dansylated phase and the two phases were gently mixed for one hour. Cyclohexane was removed and evaporated to dryness under a nitrogen stream at 60°C. The remaining precipitate was resuspended in 250 µl of a 70% acetonitrile to 30% water solution.

Prepared samples were either stored at -80 °C and assayed by HPLC at a later date or were assayed immediately. At the time of the HPLC assay, modified from the protocol described by Gerbaut., 1991 (26), 50 µl of each sample was used for injection and separated with a resolve C18 column. The mobile phase used to elute polyamines consisted of 70% acetonitrile to 30% water ramped up to 100% acetonitrile at 13 minutes, after the post-injection time, at a constant flow rate of 2 ml/min. Concentration of eluted polyamine concentration was determined according to a corresponding area under the peak, calculated by the HPLC computer. The diaminoheptane internal standard was used to normalize for possible variations in sample concentration incurred by the dansylation reaction and cyclohexane extraction method. In addition, three external standards with final concentrations of putrescine: 6, 12, 48, 72, 120 µM; spermidine: 5, 10, 20, 40, 60, 100 µM; and spermine: 3, 6, 24, 36, 60 µM were used to interpolate the polyamine concentration measured in the sample. Injections of putrescine, spermidine and spermine alone were used to determine the corresponding retention times. A standard curve was generated for each sample collected. To determine a baseline a blank solution containing 70% acetonitrile/30% water either with or without an internal standard was used prior to each assay.

5.3 RESULTS

5.3.1 *Global gene expression profiling identified relatively few genes that were differentially expressed in the unirradiated XR resistant or sensitive HCT116 cell clones:*

We have previously reported the cloning of HCT116-Clone2, HCT116-CloneK, and HCT116-Clone10 cells and their responses to both fractionated and single dose XR and, also, to various chemotherapeutic drugs such as camptothecin, topotecan, etoposide and cisplatin (14). Table 2a shows the genes that were found to be either upregulated or downregulated by more than 2 fold in the unirradiated radioresistant HCT116-Clone2 as compared to the control HCT116-Clone10. One gene was identified to be upregulated. This was an unknown expressed sequence tag (AA131311) which was up 1.7 fold (cDNA microarray) or 2.1 fold (Q-PCR). The one-downregulated gene in HCT116-Clone2 was an expressed sequence tag of unknown function. We had designated this novel EST (R40588), X-ray radiation resistance associated 1 (*XRRA1*) (GenBank BK 000541), which we found was modulated by XR treatment (27).

Table 2b shows the genes that were either upregulated or downregulated by more than 2 fold in the unirradiated radiosensitive HCT116-CloneK as compared to the control HCT116-Clone10. Four genes were identified to be upregulated. The genes of particular interest included adipose specific (*APM*) 2, which showed a 2.9 fold change by microarray but an approximate 2777 fold change by Q-PCR. The latter Q-PCR result was the average of three independent experiments. DNA electrophoresis showed that there was only one PCR product. *SSAT* had a 2.3 fold increase of expression (cDNA microarray) but 6.3 fold increase by Q-PCR. *HLA-B* histocompatibility type complex class I was up by 2.1 fold (cDNA microarray) or 4-fold (Q-PCR). Aldehyde dehydrogenase 6 was upregulated by 1.8 fold (cDNA microarray) or 2.4 fold (Q-PCR). In HCT116-CloneK, there was one-downregulated gene, cytochrome P450-CC24

(*CYP24A1*). This gene was down 2.1 fold (cDNA microarray) but approximately 1700 fold (Q-PCR). In two independent experiments, it was verified that there was only one PCR product. By considering both cDNA microarray and Q-PCR results, we found only four genes that were upregulated or downregulated by more than two fold in HCT116-Clone2 and HCT116-CloneK. These genes were *SSAT*, *APM2*, *XRRA1*, and *CYP24A1*. Since *SSAT* had a known potential link to XR response, we further analyzed the *SSAT* protein and its enzymatic activity.

5.3.2 *Protein levels and enzymatic activity of SSAT following treatment with DENSPM were relatively the highest in the unirradiated radiosensitive HCT116-Clone K:*

We used western blot analysis to determine whether the expression of *SSAT* protein levels among the unirradiated cell clones correlated with the relative basal SSAT mRNA levels of these cell clones as evaluated by cDNA microarray and Q-PCR. Basal *SSAT* protein levels in the unirradiated cells were undetectable if the cells were not treated with DENSPM. 20 μM DENSPM given for the same length of time (24 hours) induced higher levels of *SSAT* protein than 10 μM. Clearly, 10 μM of DENSPM induced the highest

Table 2. Up- or down-regulated genes with more than 2 fold change resulting from cDNA microarray analysis between (A) radioresistant cells HCT116-Clone2 versus control cells HCT116-Clone10 and (B) radiosensitive cells HCT116-CloneK versus control cells HCT116-Clone10. All errors are the standard error of the mean. cDNA microarray ratios are from three independent microarrays. Q-PCR values and standard errors are derived from the average of duplicate replicates during an independent experiment or by three independent experiments for APM2, and two independent experiments for CYP24A1 and SSAT.

Table 2A.

Genes upregulated in HCT116-Clone2 relative to HCT116-Clone10

No.	*Gene name*	*Accession No. (EST)*	*Function*	*cDNA Microarray Ratio*	*Q-PCR Ratio*
1	Unknown EST	AA131311	Unknown	1.7 ± 0.1*	2.1 ± 0.2

Genes downregulated in HCT116-Clone2 relative to HCT116-Clone10

No.	*Gene name*	*Accession No. (EST)*	*Function*	*cDNA Microarray Ratio*	*Q-PCR Ratio*
1	XRRA1	R40588	Unknown	2.2 ± 0.2	2.6 ± 1.1

*all errors represented are standard errors of the mean

Table 2B.

Genes upregulated in HCT116-CloneK relative to HCT116-Clone10

No.	*Gene name*	*Accession No. (EST)*	*Function*	*cDNA Microarray Ratio*	*Q-PCR Ratio*
1	ADIPOSE SPECIFIC 2 (APM2)	H43908	Unknown	2.9 ± 0.5*	2777 ± 318
2	SPERMIDINE/SPERMINE N1-ACETYLTRANSFERASE (SSAT)	H03112	Metabolism	2.3 ± 0.7	6.3 ± 0.1
3	HLA-B HISTOCOMPATIBILITY TYPE COMPLEX CLASS I	AA053162	Immune response	2.1 ± 0.2	4.1 ± 0.5
4	ALDEHYDE DEHYDROGENASE 6 (ADH6)	AA040747	Metabolism	1.8 ± 0.1	2.4 ± 0.2

Genes downregulated in HCT116-CloneK relative to HCT116-Clone10

No.	*Gene name*	*Accession No. (EST)*	*Function*	*cDNA Microarray Ratio*	*Q-PCR Ratio*
1	CYTOCHROME P450-CC24 (CYP24A1)	H96028	Metabolism	2.1 ± 0.4	1726 ± 108

*all errors represented are standard errors of the mean

level of *SSAT* in HCT116-CloneK and HCT116-Clone2. By contrast, HCT116-Clone10 demonstrated the least degree of response to DENSPM (Fig. 1). The latter was most evident when referring to the lack of induction of *SSAT* by 10 μM DENSPM in HCT116-Clone10 whereas this concentration of DENSPM already induced detectable amounts of *SSAT* in HCT116-CloneK, HCT116-Clone2 and the parental HCT116 cells. At 20 μM DENSPM, *SSAT* protein levels were found to be maximally expressed for all of the cell clones except for HCT116-Clone10 which at this concentration is barely detectable.

We then determined the relative enzymatic activity of *SSAT* in the unirradiated clones. We found that without DENSPM, *SSAT* activity was at background levels and thus not distinguishable among the cell clones. Therefore, we normalized the *SSAT* activity without DENSPM to "100". Following treatment with 20 μM DENSPM for 24 hours, it was again evident that HCT116-CloneK showed the highest amount of response (upregulated about 22-fold compared to the basal level), whereas HCT116-Clone10 showed the lowest amount of response (upregulated only about 4-fold from basal level) to DENSPM (Fig. 2). HCT116-Clone2 showed relatively lower SSAT activity (~ 7 fold) despite showing high protein levels by western immunoblotting suggesting at least some of the SSAT induced in HCT116-Clone2 was inactive. Interestingly, parental HCT116 cells responded more similarly to both HCT116-Clone10 and HCT116-Clone2 than HCT116-CloneK based on the western blot (Fig. 1) and activity (Fig. 2) results.

Figure 1. Dose dependent expression of *SSAT* protein following induction with DENSPM in HCT116-Clone10, HCT116-Clone2, HCT116-CloneK and parental HCT116 cells. Cells were incubated at 37°C with the indicated concentrations of DENSPM for 24 hours. Then, western blot was carried out using a rabbit polyclonal *SSAT* antibody to recognize a band 20 kDa in size. Total protein loaded onto each lane was 50 μg. Beta actin (42 kDa) was used as loading control and SSAT protein levels were normalized to beta actin protein levels. HCT116-CloneK contained the highest level of *SSAT* after induction with 10 μM DENSPM.

Figure 1.

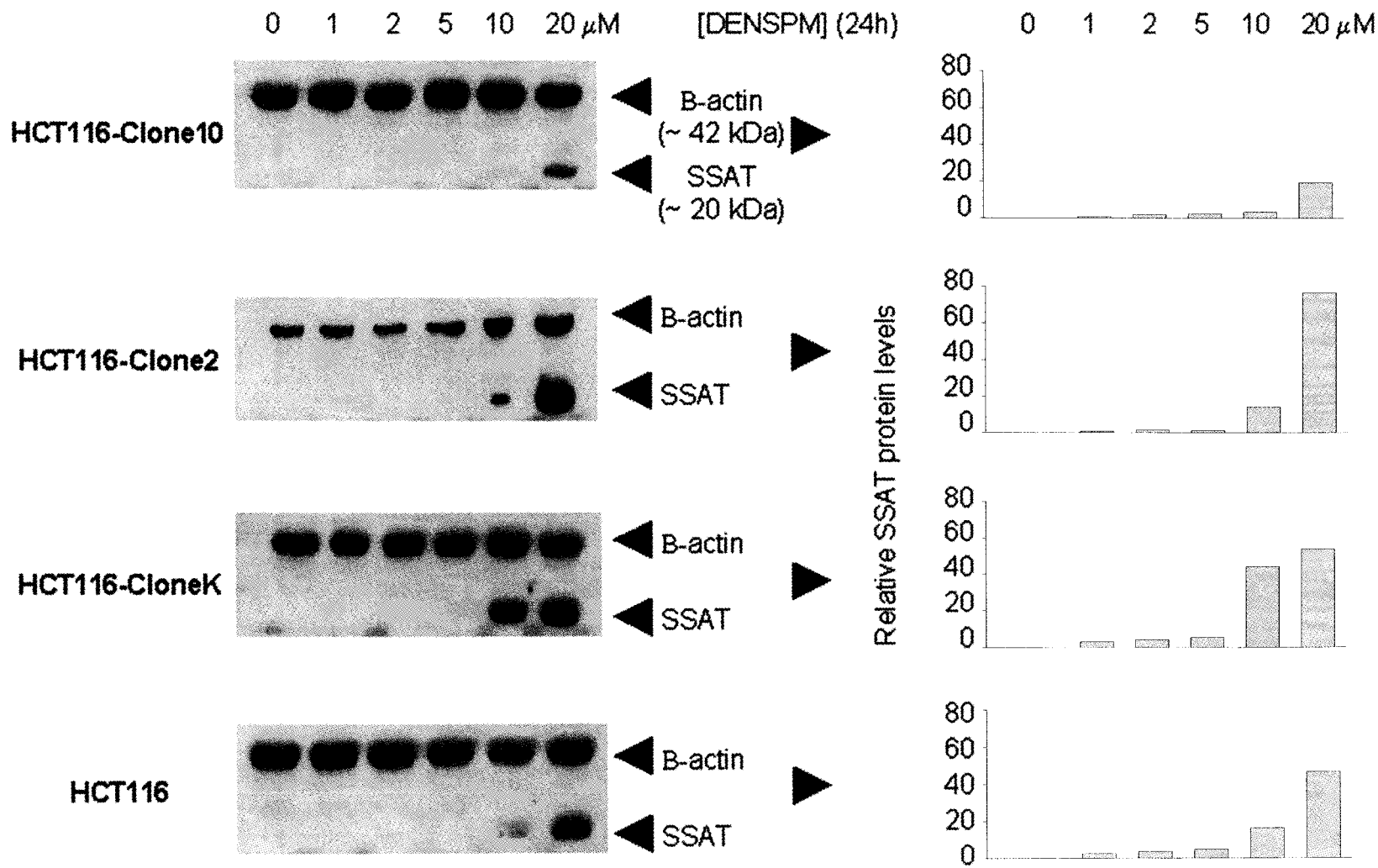
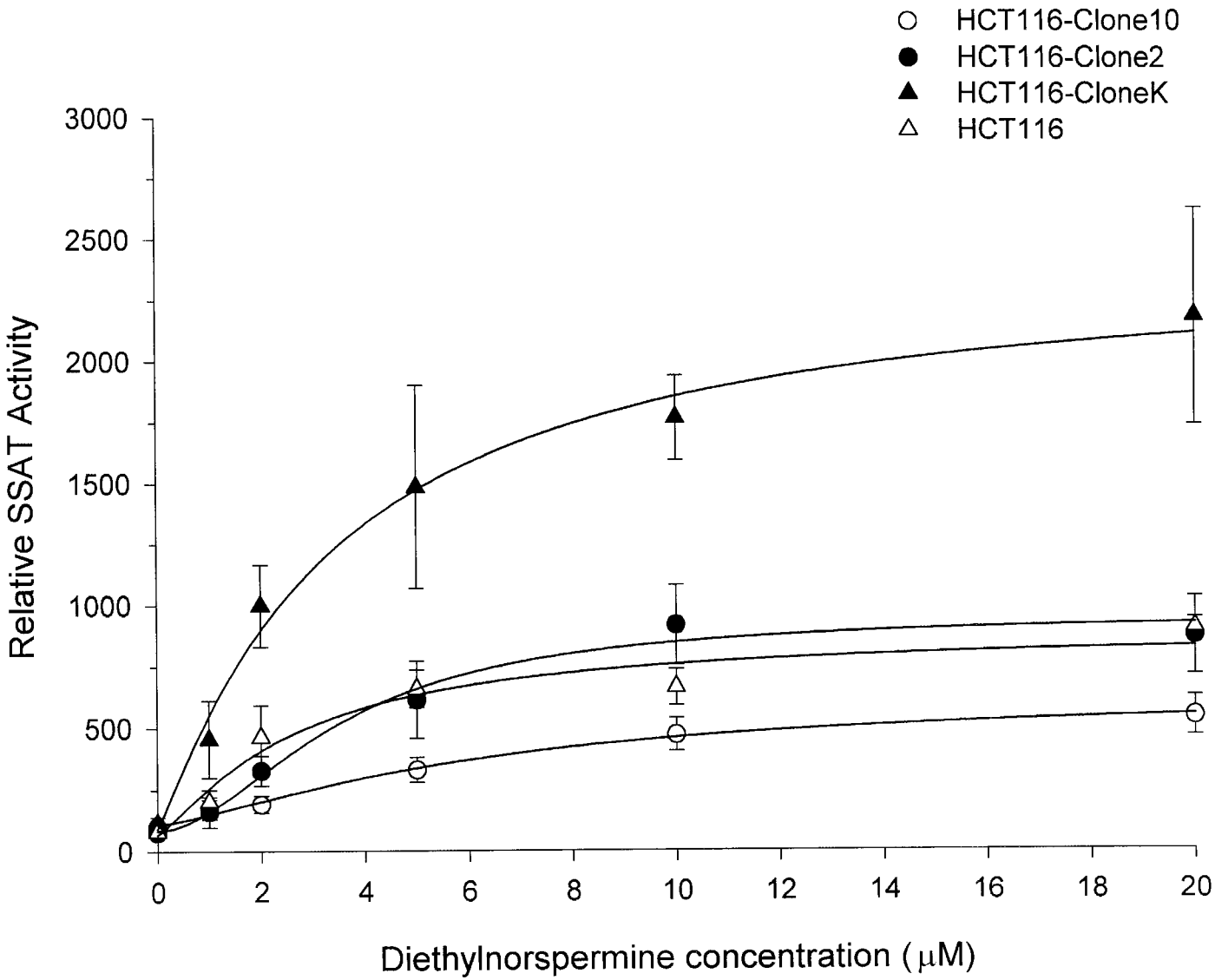

0 1 2 5 10 20 μM
[DENSPM] (24h)
HCT116-Clone10
B-actin
(~ 42 kDa)
SSAT
(~ 20 kDa)
HCT116-Clone2
B-actin
SSAT
HCT116-CloneK
B-actin
SSAT
HCT116
B-actin
SSAT
Relative SSAT protein levels
80
60
40
20
0

Figure 2. Relative enzymatic activity of *SSAT* cell clones following induction with DENSPM in HCT116-Clone10, HCT116-Clone2, HCT116-CloneK and parental HCT116 cells. Cells were incubated at 37°C with the indicated concentrations of DENSPM for 24 hours. Then, enzymatic activity was measured by the rate of incorporation of a radiolabelled acetyl group from acetyl CoA to the polyamines spermidine and spermine. Values shown represent triplicate experiments. HCT116-CloneK contained the highest level of *SSAT* activity after induction with DENSPM.

Figure 2.


HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
Relative SSAT Activity
3000
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
16
18
20
Diethylnorspermine concentration (μM)

5.3.3 *Spermidine (SPD) was relatively the lowest of the polyamine levels in the HCT116 cell clones following treatment with DENSPM and/or XR:*

Because *SSAT* back-converts spermine (SPM) to spermidine (SPD) and the latter to putrescine (PUT), we evaluated the relative intracellular levels of these polyamines in the unirradiated cell clones. With the exception of HCT116-Clone2, the other cell clones as well as the parental HCT116 cells contained about the same basal (i.e. untreated) levels of cellular polyamines as determined by HPLC (Fig. 3). Basal levels of SPM were 1.5 and 3-4 times relatively more abundant than SPD and PUT, respectively (Fig. 3). HCT116-Clone2 had the highest relative basal abundance of SPM and SPD ($p < 0.01$) but not PUT. There were no significant differences in PUT levels before or following treatment with 10 μM DENSPM, as well as 4 Gy XR or combined DENSPM and XR (Fig. 3a). SPD content was found to be significantly higher in the untreated HCT116-Clone2 than the other untreated clones ($p < 0.01$). Treatment with DENSPM decreased SPD by 3-4 fold ($p < 0.05$) whereas 4 Gy XR decreased the SPD by about 1.5 fold but was not significant accordingly to the Bonferroni multiple comparisons post test (n=3-4) (Fig. 3b). Interestingly, a highly significant decrease in SPD levels was observed in HCT116-Clone2 after DENSPM treatment ($p < 0.001$) and combined treatment of DENSPM and XR almost eliminated the cellular SPD in the parental cell line and its sublines, especially HCT116-CloneK (Fig. 3b). Similar to SPD levels, SPM content amongst the untreated cells was the highest in HCT116-Clone2 and treatment with DENSPM significantly lowered SPM levels ($p < 0.05$) chiefly in DENSPM-treated HCT116-Clone2 ($p < 0.01$) and when combined with XR ($p < 0.05$) but not significantly

for treatment with XR alone (Fig. 3c). Lastly, total polyamine levels were also discovered to be significantly higher in untreated HCT116-Clone2 than in the untreated parental cell line and its sublines ($p < 0.05$). Similarly, the combination of DENSPM and XR significantly lowered the collective polyamine levels to approximately 10 μM / mg protein of total intracellular polyamine concentrations in all of the cell clones with the most significant decrease in HCT116-Clone2 at a p-value of 0.001 (Fig. 3d). Therefore, it is clear that both SPM and SPD but not PUT levels are decreased by both DENSPM and XR and that almost complete depletion of SPM and SPD was achieved by combined DENSPM and XR treatments. Furthermore, the data clearly shows that the radioresistant HCT116-Clone2 contained the highest polyamine concentration and was the most significantly affected by DENSPM treatment.

Figure 3. Polyamine pool in cell clones HCT116-Clone10, HCT116-Clone2, HCT116-CloneK and parental HCT116 cells with or without DENSPM or XR or both DENSPM and XR as measured by HPLC. (A) Treatment with 10 μM DENSPM for 24 hours, or XR at 4 Gy, or combined DENSPM and XR did not significantly decrease PUT levels in all cell clones ($p > 0.05$). (B) SPD content was significantly higher in untreated HCT116-Clone2 than untreated HCT116-Clone10, HCT116-CloneK ($p < 0.01$) and parental HCT116 ($p < 0.05$). Treatment with 10 μM DENSPM for 24 h significantly decreased SPD levels in the cell clones as compared to the untreated cells ($p < 0.05$) particularly HCT116-Clone2 ($p < 0.001$). XR treatment alone did decrease SPD levels by about 1.5 fold but was not significant, however, combined treatment with DENSPM and XR reduced SPD to almost undetectable levels (i.e. HCT116-CloneK). (C) Similarly, SPM content was higher in untreated HCT116-Clone2 than untreated HCT116-Clone10, HCT116-CloneK and parental HCT116 but was not statically significant. However, SPM was dramatically decreased in the presence of DENSPM ($p < 0.05$) especially in HCT116-Clone2 ($p < 0.01$) and when combined with XR ($p < 0.05$) but not XR alone. (D) Collective polyamine levels were also significantly higher in untreated HCT116-Clone2 than untreated clones ($p < 0.05$). Correspondingly, total polyamine concentration decreased in all of the cell clones in the presence of DENSPM ($p < 0.05$) particularly HCT116-Clone2 ($p < 0.001$) and when combined with XR ($p < 0.05$). *p*-values were determined by the one-way analysis of variance (ANOVA) with the Bonferroni multiple comparisons post test. Each bar represents the mean and standard error of three to four independent experiments.

Figure 3a.

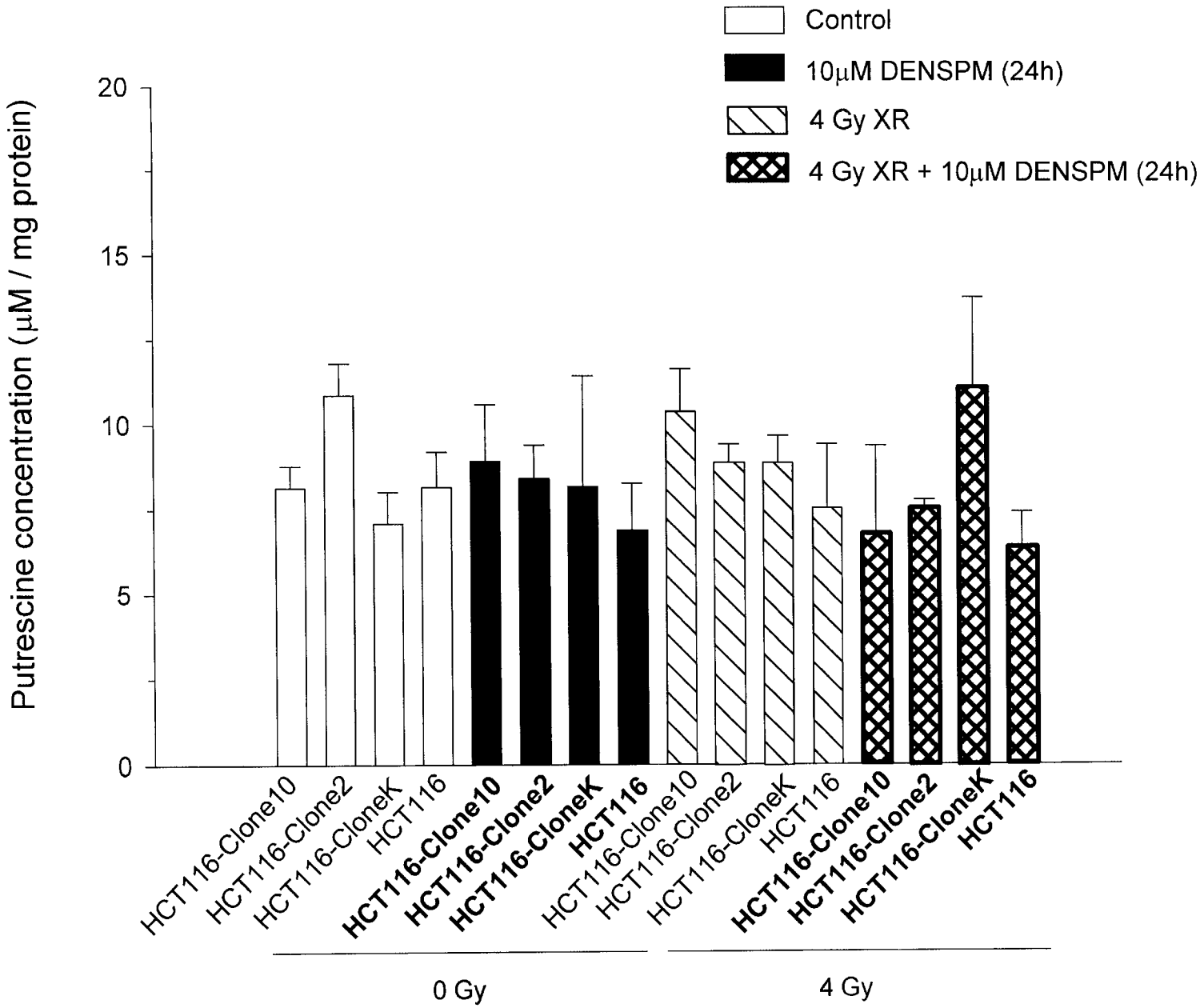

Control
10μM DENSPM (24h)
4 Gy XR
4 Gy XR + 10μM DENSPM (24h)
Putrescine concentration (μM / mg protein)
20
15
10
5
0
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
0 Gy
4 Gy

Figure 3b.

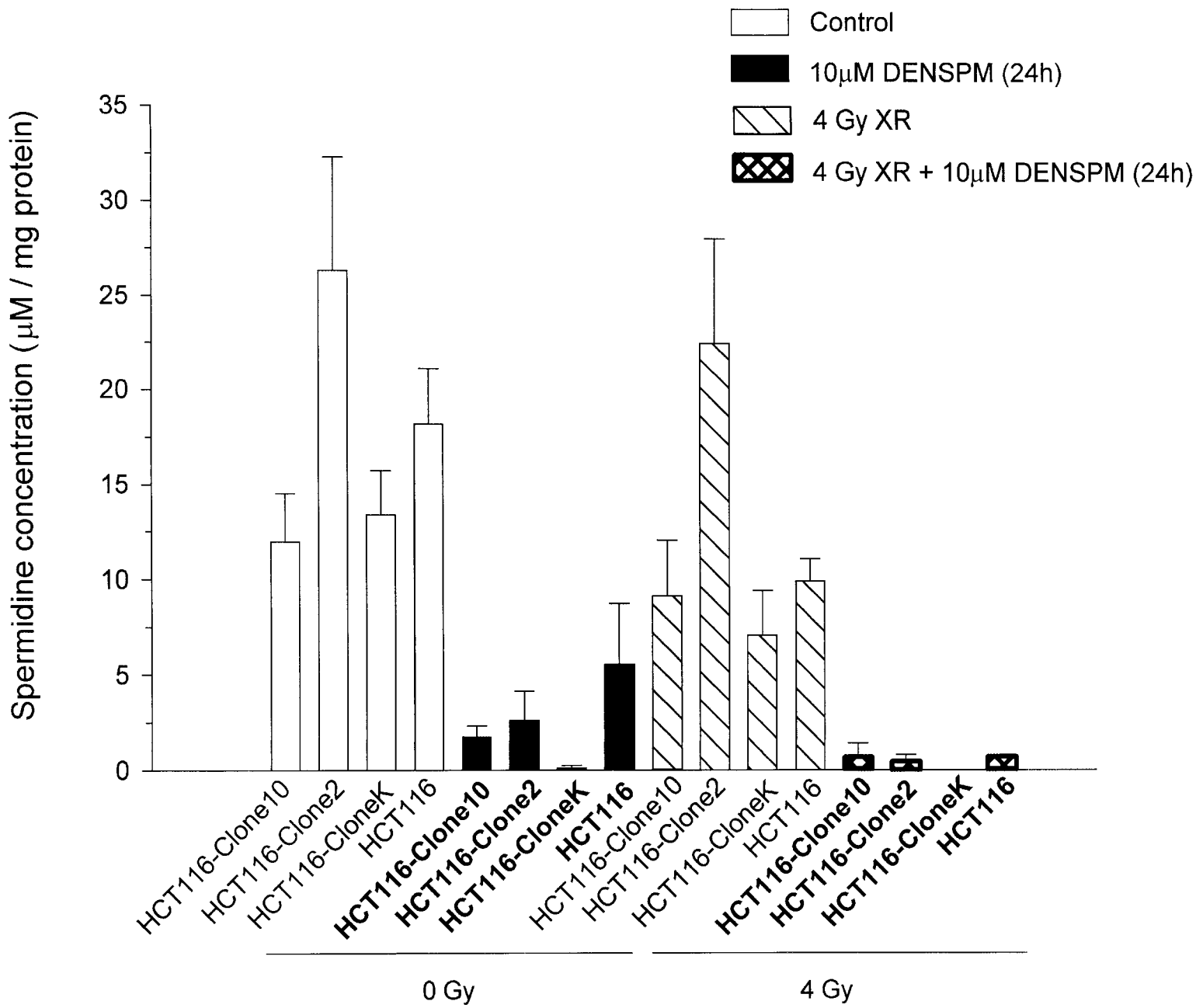
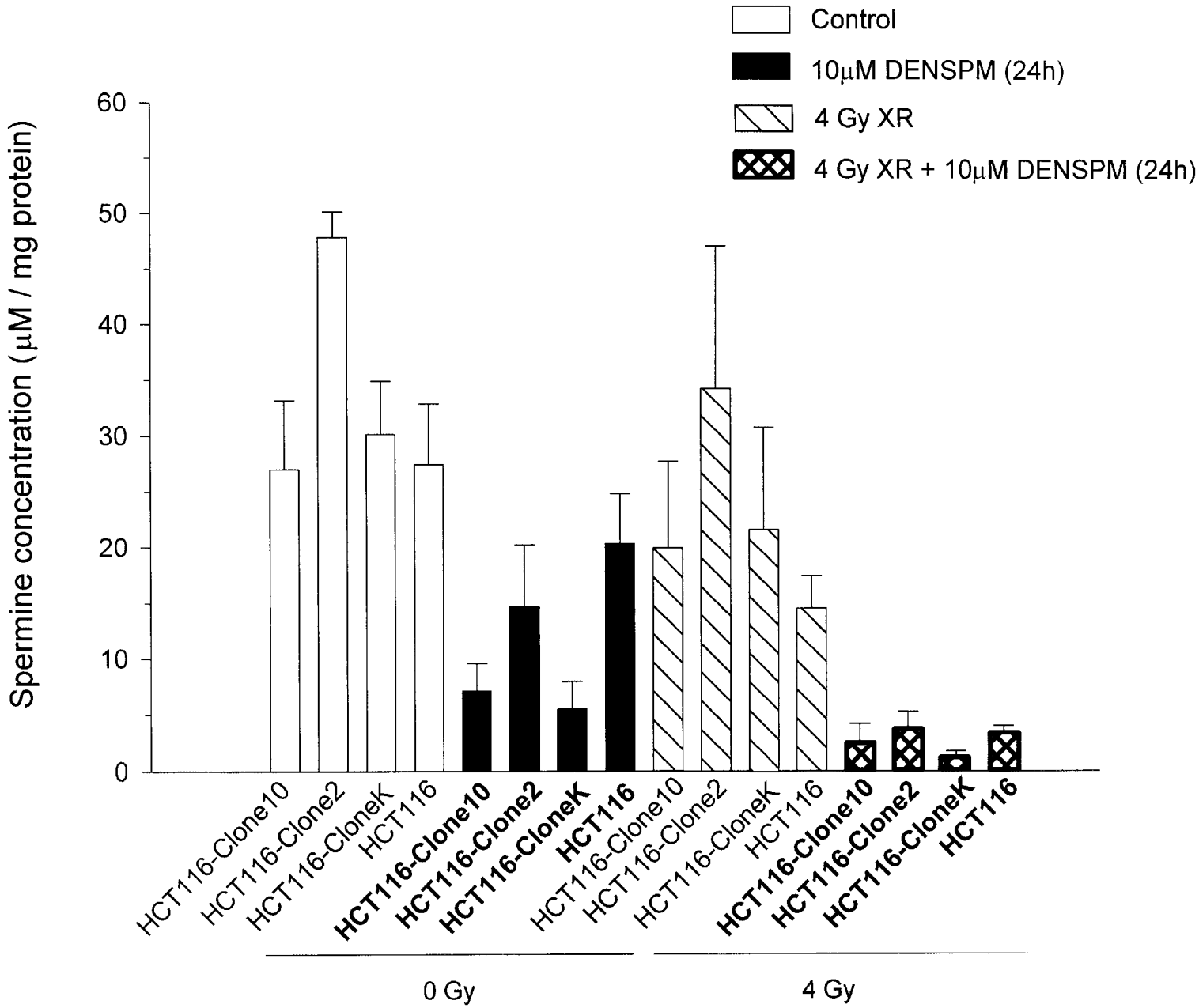

Control
10μM DENSPM (24h)
4 Gy XR
4 Gy XR + 10μM DENSPM (24h)
Spermidine concentration (μM / mg protein)
35
30
25
20
15
10
5
0
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
0 Gy
4 Gy

Figure 3c.

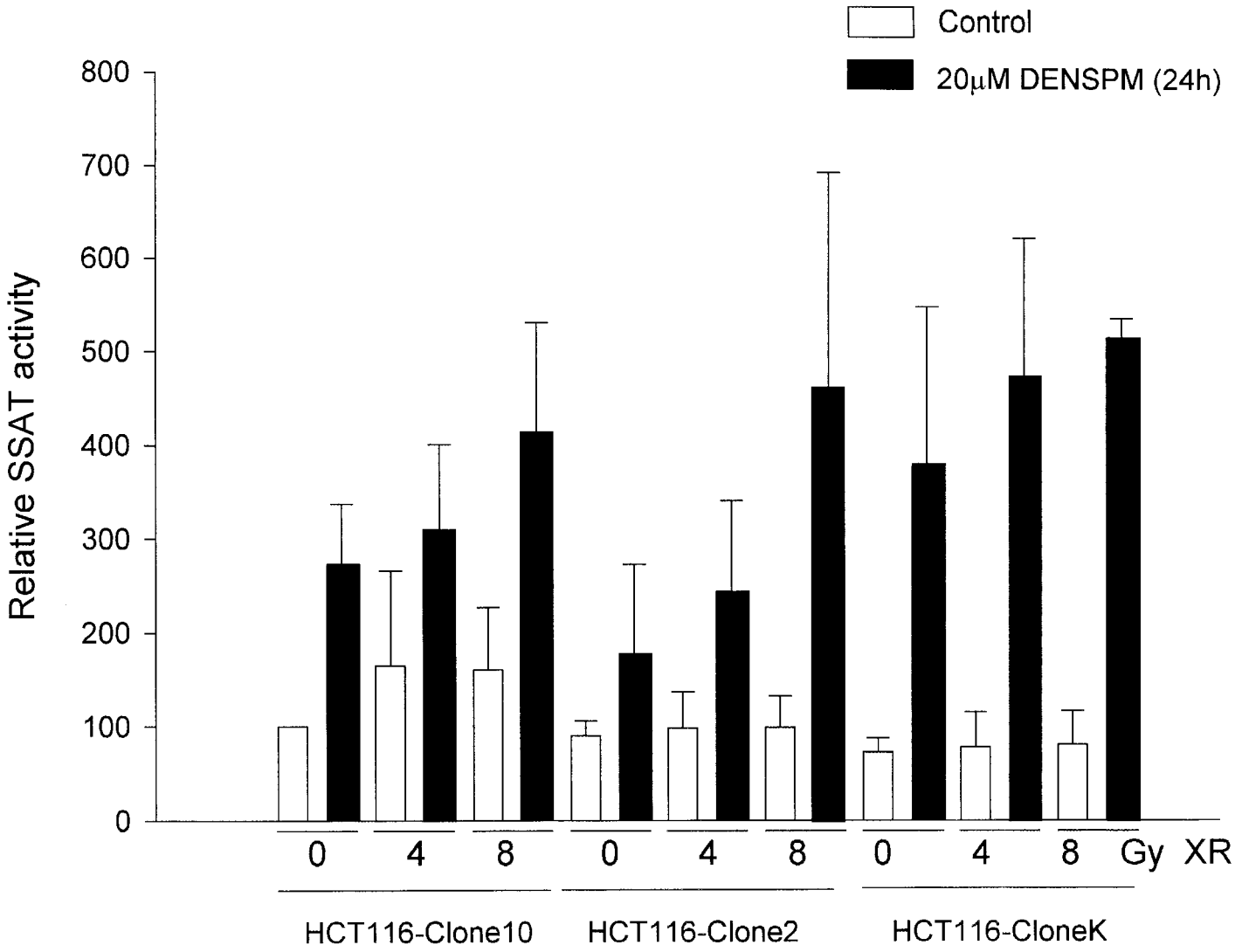

Control
10μM DENSPM (24h)
4 Gy XR
4 Gy XR + 10μM DENSPM (24h)
Spermine concentration (μM / mg protein)
60
50
40
30
20
10
0
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
0 Gy
4 Gy

5.3.4 *Lack of induction of SSAT enzymatic activity by XR showed no correlation to the relative XR response of the cell clones:*

We next investigated whether XR stress can affect the enzymatic activity of *SSAT* in these cell clones and whether there was any correlation with the relative sensitivity/resistance exhibited by the cell clones. DENSPM was again used to permit the changes in *SSAT* levels to be detectable. We have determined that in the presence of DENSPM, XR stress was unable to significantly increase *SSAT* activity in all three cell clones ($p > 0.05$) as determined by the one-way analysis of variance (ANOVA) with the Bonferroni multiple comparisons post test (Fig. 4). Thus, following XR, the lack of induction of *SSAT* enzymatic activity did not correlate with the relative XR response of the cell clones. It is likely that the radiation response of the cell clones, in particular HCT116-Clone2, may be due to the depletion of both SPM and SPD in the presence of DENSPM and that other mechanisms that regulate polyamine levels, including *SSAT*, may be involved.

Figure 4. Effects of XR on *SSAT* enzymatic activity. Cells were treated with XR and incubated 37°C in the absence or presence of 20 μM DENSPM for 24 h, and then *SSAT* expression was determined with the activity assay. Note that control cells were not given DENSPM. Relative enzymatic activity of *SSAT* after 4 or 8 Gy XR in the presence or absence of DENSPM. Although there was increasing *SSAT* activity with increasing XR doses, *SSAT* activity was not significantly different in all cell clones. Each bar represents the mean and standard error of three independent experiments.

Figure 4.


Control
20μM DENSPM (24h)
Relative SSAT activity
800
700
600
500
400
300
200
100
0
0
4
8
0
4
8
0
4
8
Gy XR
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK

5.3.5 *Treatment with both XR and DENSPM in comparison to XR alone was associated with decreased cellular survival and significant radiosensitization in all the cell clones except for radiosensitive HCT116-CloneK:*

Since initial experiments demonstrated that the combination of XR at 4 Gy and 20 μM DENSPM led to very high cell killing, these experiments were done with 10 μM DENSPM instead. Ten μM of DENSPM given without XR resulted in about 40 – 60 % clonogenic cell kill in all cell clones (Fig. 5). When DENSPM was given together with 4 Gy XR, there was substantially increased clonogenic cell killing in all cell clones, particularly in HCT116-clone2 ($p < 0.001$), except in HCT116-CloneK when compared to XR alone (Fig. 5). A significant sensitizer enhancement ratio (SER) was also found with the combination of DENSPM (10μM) and XR treatment in all the cell clones, especially HCT116-Clone2, but not in HCT116-CloneK (Table 3). This suggested that the DENSPM induced *SSAT* enzymatic activity, which significantly decreased endogenous levels of SPM and SPD, was responsible for the radiosensitization of HCT116, HCT116-Clone10, and HCT116-Clone2, but not HCT116-CloneK.

Figure 5. Clonogenic surviving fraction of HCT116-Clone10, HCT116-Clone2, HCT116-CloneK and parental HCT116 cells with or without 4 Gy of XR in the absence or presence of 10 μM DENSPM. DENSPM decreased surviving fraction for each cell clones with HCT116-CloneK showing the lowest relative survival. XR treatment at 4 Gy reflected the differences of XR responses among the HCT116 cell clones i.e. HCT116-Clone 2 or HCT116-CloneK was relatively radioresistant or radiosensitive, respectively, compared to parental HCT116 or HCT116-Clone10. Surviving fraction after XR in the presence of DENSPM was normalized to the one with DENSPM but without XR. Each bar represents the mean and standard error of three independent experiments.

Figure 5.

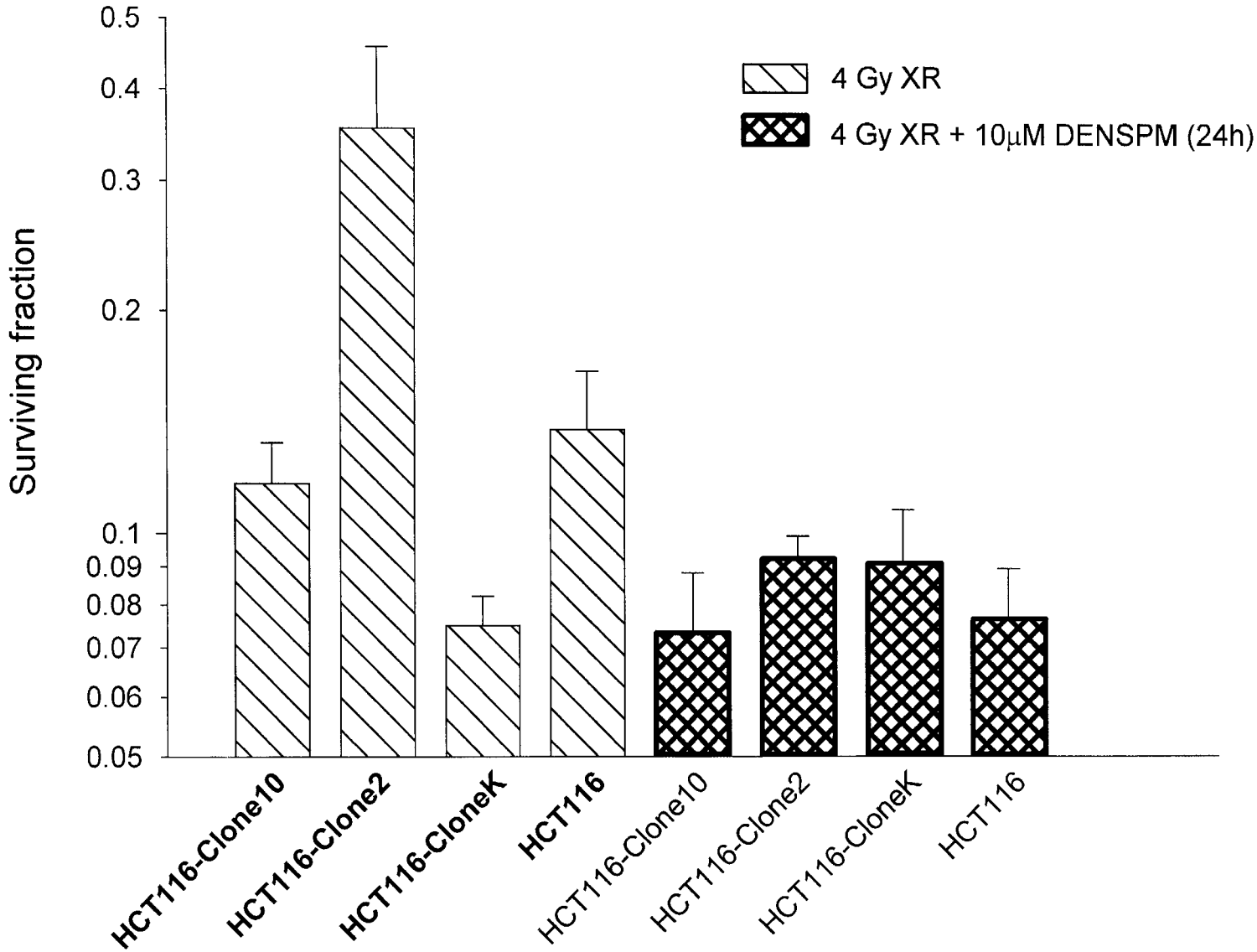

Surviving fraction
0.5
0.4
0.3
0.2
0.1
0.09
0.08
0.07
0.06
0.05
4 Gy XR
4 Gy XR + 10μM DENSPM (24h)
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116
HCT116-Clone10
HCT116-Clone2
HCT116-CloneK
HCT116

Table 3. Treatment with DENSPM was associated with radiosensitization (expressed as sensitizer enhancement ratios (SER)) in the HCT116 parental cell line and all of the cell clones, except for radiosensitive HCT116-CloneK. Values shown represent three independent experiments.

Table 3.

Cell line	*Plating efficiency %*	*Effect of DENSPM (10 μM – 24h) on survival of the non-X-radiation treated cell lines*	*SER at 4 Gy*
HCT116-Clone10	40.7 ± 4.5	0.57 ± 0.10	1.60
HCT116-Clone2	38.0 ± 5.5	0.60 ± 0.18	3.66
HCT116-CloneK	38.7 ± 2.6	0.44 ± 0.11	0.82
HCT116-Parental	55.3 ± 5.5	0.53 ± 0.17	2.07

All errors are the standard of the mean

NOTE: To distinguish drug alone effects all plating efficiencies were normalized to one

Table 3.

Cell line	*Plating efficiency %*	*Effect of DENSPM (10 μM – 24h) on survival of the non-X-radiation treated cell lines*	*SER at 4 Gy*
HCT116-Clone10	40.7 ± 4.5	0.57 ± 0.10	1.60
HCT116-Clone2	38.0 ± 5.5	0.60 ± 0.18	3.66
HCT116-CloneK	38.7 ± 2.6	0.44 ± 0.11	0.82
HCT116-Parental	55.3 ± 5.5	0.53 ± 0.17	2.07

All errors are the standard of the mean

NOTE: To distinguish drug alone effects all plating efficiencies were normalized to one

5.4 DISCUSSION

The studies described in this paper address an important question that has hitherto not been discussed: can gene expression profiling of unirradiated HCT116 cell clones displaying either the XR-radiation resistant or sensitive response identify genes that are relevant to their response to XR? We used cDNA microarrays containing approximately 19,200 ESTs that would represent roughly one third of the total human genes (28) to investigate the transcriptomic differences among our XR-resistant or sensitive clones. It is worthwhile noting that only Kitahara et al. (5) have screened a number of genes in cervical squamous cell carcinoma that is comparable to our study and that the other studies have considerably screened fewer genes. One interesting feature of our study was that we had identified relatively few genes that were differentially expressed either in the unirradiated XR-resistant or sensitive cell clones when compared to the control cell clone HCT116-Clone10. This result was actually expected since these cell clones were derived from the parental HCT116 and were, therefore, genetically similar. Thus, cDNA microarray analysis had further verified the G-banding analysis of these HCT116 cell clones that we had published earlier (14).

The majority of genes that we measured with the cDNA microarrays were differentially expressed by less than 2 fold. We tabulated genes that had a cDNA microarray ratio of at least 1.5 but only displayed data that had changed at least 2 fold based on more reliable Q-PCR analysis (Table 2). Except for *SSAT*, none of the other genes was either known to be associated with or putatively linked to XR responses involving DNA damage sensor/repair, cell cycle arrest or apoptosis. Some of the genes

were ESTs of unknown functions (including *APM2*), while others were linked with signaling or various metabolic activities (*SSAT*, *ADH6*, *CYP24A1*).

The discrepancy with the genes *APM2* and *CYP24A* between the microarray and Q-PCR may indicate a flaw in the array in that it may have decreased sensitivity. Therefore, cDNA microarray results need to be validated with other independent gene expression techniques such as Q-PCR, Northern blot or RNase protection assay (29). After verifying with Q-PCR, we found only four genes that were differentially expressed by more than 2 fold with both cDNA microarray and Q-PCR. These genes were *APM2*, *XRRA1*, *SSAT*, and *CYP24A1*. We further evaluated the transcript levels of *APM2*, *XRRA1*, and *SSAT* after XR at different doses and times following XR. *APM2* was consistently significantly upregulated in the radiosensitive HCT116-CloneK relative to the control cell clone HCT116-Clone10 (data not shown). By contrast, *XRRA1* message was differentially modulated according to XR doses and length of time after XR (data not shown). Both *APM2* and *XRRA1* are novel genes with unknown functions. Only *SSAT*, as described earlier, had a potential link to XR response (24). In addition to identifying genes that are potentially involved in XR response, our studies here also provided the first evidence suggesting a potential role for employing the expression of *SSAT* gene to predict cellular response to XR.

The higher abundance of *SSAT* mRNA in the unirradiated, radiosensitive HCT116-CloneK relative to control HCT116-Clone10 corresponded with the increased XR sensitivity of these cells. Unfortunately, the *SSAT* protein as well as the enzymatic activity in all the un stimulated HCT116 cell clones were below detectable levels (Fig. 1, 2), thus preventing us from correlating the endogenous protein levels of *SSAT* with XR response. The low basal *SSAT* protein levels were likely due to the rapid turnover of

SSAT protein ($t_{1/2}$ of 29 min) associated with its degradation through the ubiquitin/proteasomal pathway (30). Employing small interfering RNA to knock down the *SSAT* mRNA would probably not be useful for demonstrating an effect on XR response. Therefore, we were left with three options to address this issue: using the proteasomal inhibitor MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal), over-expressing *SSAT* or employing polyamine analogues such as N^1, N^{11}-diethylnorspermine (DENSPM) as inducers of *SSAT*. DENSPM inhibits the ubiquitination of *SSAT* and prevents its targeting to the proteasome (31). Over-expressing *SSAT* was hampered by the inability to obtain efficient translation of the transfected *SSAT* cDNA in human cells (32). Successful stable transfection into Chinese hamster ovary, breast cancer MCF-7 and, recently, into small cell lung carcinoma cells, achieved only moderate elevation of *SSAT* activity (30, 33, 34). Hence, DENSPM was still needed to boost the enzymatic activity levels of *SSAT* to assist experimental measurement. Since MG132 would not only have lengthened the half life of *SSAT* protein, but that of other short-lived molecules as well, we opted to use DENSPM with the assumption that this analogue will affect all HCT116 cell clones similarly. Thus, we examined the combined modalities of DENSPM and XR on the XR response among these cell clones.

Consistent with other studies (33, 35, 36), 10 μM DENSPM given for 24 hours substantially decreased cellular contents of SPD and SPM. Unlike some of the other studies, however, PUT did not change in all HCT116 cell clones 24 hours after the treatment (Fig. 3). For example, Niiranen K et al. observed 20-30% increase in PUT and N^1-acetylspermidine 48 hours after 50 μM DENSPM treatment in mouse embryonic stem cells RW-4 (35). Hegardt C et al. (36) showed approximately four fold decrease of PUT level in breast cancer cell L56Br-C1 24 hours after treatment with 10 μM DENSPM. In

another study with wild type p53 melanoma cells, PUT content was 4.5 fold less, whereas in another melanoma cell line with mutated p53, the PUT level was 3.5 times higher after treatment with 10 μM DENSPM. Besides inducing *SSAT*, DENSPM down regulates two other enzymes involved in polyamine metabolism, ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) (37). Therefore, there will be little or no new putrescine entering the cellular polyamine pool resulting from biosynthetic activities of these two enzymes. The lack of an increase in PUT content in all HCT116 cell clones following DENSPM induction suggested a low efficiency in the backconversion of SPM to SPD and then into PUT probably because the acetylated forms of SPM and SPD resulting from the DENSPM-induced high *SSAT* activities were being exported out of the cell rather than being converted into PUT.

Interestingly, combined treatment with DENSPM and XR, almost depleted the cellular content of SPD and SPM but not PUT. Since PUT levels remained steady, the depletion of SPD and SPM, as shown by Figure 3, reflected *SSAT* activity in these HCT116 cell clones. As a consequence of the diminished SPD and SPM contents, we did not detect a significant difference in *SSAT* activity among the HCT116 cell clones (Figure 4). By contrast, with DENSPM alone (Fig. 2) we were able to detect different *SSAT* activity level in HCT116 cell clones according to their XR response (i.e. radiosensitive cells HCT116-CloneK contained the highest *SSAT* activity). This data was also supported by the clonogenic assay in the presence of either DENSPM alone or both DENSPM and XR. Addition of 10 μM DENSPM led to 40-60% cell killing with HCT116-CloneK being relatively the most sensitive compared to the other HCT116 cell clones (Table 3). It has been known that DENSPM induces rapid caspase-dependent cell death (36). Cell killing was continued in the presence of both DENSPM and XR for all cell clones except

radiosensitive HCT116-CloneK. The surviving fraction of HCT116-CloneK was not changed in the presence of both agents, perhaps because the higher *SSAT* activity had depleted the SPM and SPD faster in this clone than in the other cell clones. The lack of radiosensitization of HCT116-CloneK does suggest that polyamines may not play a significant role in its radioresponse and that other mechanisms may be involved. Albeit, we do show a strong correlation with the significant relative radioresistance exhibited by HCT116-Clone2 and its relatively high endogenous polyamine levels (SPM and SPM) as compared to the other clones. Further evidence of the role in polyamines in the radioresponse of HCT116-Clone2 is that its relative radioresistance is eliminated when XR is combined with DENSPM. In addition, HCT116-Clone10 and the parental HCT116 cell line also show radiosensitization in the presence of DENSPM along with decreases in SPN and SPD content and an increase in *SSAT* activity.

In conclusion, our data suggested that *SSAT* mRNA subsequent to DENSPM induction of HCT116 cell clones corresponded positively with the XR response of the radiosensitive HCT116-CloneK. Basal gene expression did predict the increased levels of *SSAT* protein and enzymatic activity observed in HCT116-CloneK relative to the other clones in the presence of DENSPM.

However, the lack of radiosensitization by DENSPM in HCT116-CloneK shows that *SSAT* function may not be the only contributing factor in the radioresponse and should be looked at as a potential indicator of radioresponse in relation to other regulators of polyamine synthesis and metabolism (i.e. ODC). Interestingly, combined modality treatment of DENSPM and XR did enhance cell killing of HCT116-Clone10, HCT116-Parental, and, in particular, significantly increased the radiosensitization of radioresistant HCT116-Clone2 which correlated with the enhanced *SSAT* activity and the depletion of endogenous radioprotective polyamines. This suggested that combined DENSPM and XR treatment may be a useful chemoradiation strategy. In fact, DENSPM has been tested in clinical trials in patients with lung cancer with minimal toxicity (38). Similarly, a phase II study with DENSPM in patients with metastatic breast cancer has shown the drug to be quite tolerable but has been shown to be ineffective as a stand-alone agent (39). In either of these studies the use of DENSPM was independent of XR treatment and failed to examine the effectiveness of DENSPM in combination with tradition radiation therapy, a strategy that we demonstrate to hold the most potential for radiosensitization. Finally, *SSAT* could potentially be useful as a marker for predicting cellular response to XR and genome-wide profiling of gene expression in unirradiated tumour cells may be useful for identifying genes that can affect their XR responses.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

6. GENERAL DISCUSSION

6.1 *Brief overview*

The literature review has led us to conclude that the mechanisms of radioresistance are complex and may not be isolated strictly to the existing mechanisms that are known to be associated with radiosensitization. These mechanisms include alterations in efficiency of DNA repair, enhanced apoptosis, or cell cycle perturbations, but may also involve a number of novel mechanisms previously thought not to be associated with the response of cells to radiation that are described herein. To recapitulate, the purpose of this study was to understand the contribution of intrinsic cellular factors that govern cellular radioresponsiveness as well as address the ability of particular chemotherapeutic agents to enhance radiosensitivity. To address this goal, we had pursued four main objectives as previously mentioned at the end of the first chapter.

6.1.1 *General discussion on understanding the mechanism of cellular death of human tumour and normal cells following treatment to CPT and X-radiation*

The first objective, in Chapter 2, addressed identifying the mechanisms of cellular death of a radioresistant human tumour cell line and normal cell line following treatment to CPT and X-radiation. We have shown that variations in cytotoxic effects induced by X-radiation and the topoisomerase-I poison, camptothecin (CPT) in human melanoma and normal human fibroblasts, may be mediated through differences in the amount and/or

rate of induction of apoptotic and necrotic death. After treatment with CPT alone, both apoptotic and necrotic cell death increased significantly in the radioresistant Sk-Mel 3 melanoma cell line relative to untreated controls. Moreover, combination of these two agents was found to increase necrotic cell death more so than apoptotic death in the melanoma cell line. Neither of these agents had a significant effect on either apoptosis nor necrosis in fibroblast (AG1522) cells. Clonogenic assays for the melanoma cell line were in agreement with the apoptotic/necrotic assays demonstrating that these agents either given separately or concurrently led to a substantial increase in cell death.

The data in Chapter 2 illustrate the importance of apoptosis and necrosis in combinational chemo- and radio-therapy. Recognizing the contribution of these classes of cell death in combinational treatment can potentially be useful in minimizing normal tissue damage and yet maximize the killing of tumour tissue thus providing possible relevance to the clinical setting. We have shown that necrotic cell death plays a very important role in the killing of melanoma cells when exposed to CPT and IR, but not in the human fibroblast cell line. As mentioned in Chapter 2, the advent of apoptosis and necrosis are not necessarily two independent events and can share a common pathway. This phenomenon has been termed by Lemasters in 1999 as necrapoptosis (Lemasters, 1999). Necrapoptosis can be mediated by a number of events. For example, disruption of a kinase chaperone, heat shock protein 90 (HSP90), has shifted the tumour necrosis factor (TNF) initiated cell death response of a murine fibrosarcoma (L929sA) cell line from necrosis to apoptosis. Several lines of evidence from this study suggested that the availability of this stressor adaptor protein determines the cell death pathway via death

receptors. The role of necrapoptosis, in this model, has been suggested to provide a backup suicide program in cells that possess defective apoptotic signaling proteins which are commonly seen in tumour or virally infected cells (Vanden Berghe *et al.*, 2003). Therefore, it would be interesting to promote the expression of HSP90 in our system as we may thereby exploit the sensitivity of melanoma cells to necrototic cell death and promote additional tumour cell death or in any other tumour cell line that may be resistant to apoptosis.

Necrapoptosis is also prevalent in tissues experiencing acute metabolic perturbations due to permeabilization of the mitochondrial inner membrane. Conditions such as this are commonly seen within tumour tissue (Kizaka-Kondoh *et al.*, 2003; O'Brien *et al.*, 1997; Steen, 1991) and are also of particular interest in our study. Factors, such as reactive oxygen species, ischemia followed by reperfusion, tumour necrosis factor-alpha exposure, Fas ligation, inorganic phosphate, and increases of mitochondrial calcium can promote mitochondrial dysfunction or mitochondrial permeability transition (MPT) and increase mitochondrial inner membrane permeability. Signalling of apoptosis or necrosis after the onset of MPT is dependant on intracellular levels of adenosine triphosphate (ATP) (Lemasters, 1999). For MTP to cause apoptosis, ATP has to be readily available since it is an active process requiring energy. Thus, if the metabolic perturbations caused by MTP are large enough to deplete ATP stores significantly, then the prevalent mode of cell death is by necrosis and may also be present in our system.

Lastly, the proapoptotic protein Bax has also been attributed to necrapoptosis (Cheng *et al.*, 2003). Analogous to most pro-apoptotic members of the Bcl-2 family, Bax elicits cell death via mitochondrial membrane permeabilization by forming ion channel complexes and disrupting cellular calcium homeostasis whereas Bcl-2 maintains the mitochondrial membrane barrier function and inhibits the formation of the permeability transition pore complex by Bax (Brenner *et al.*, 2000; Nutt *et al.*, 2002a; Nutt *et al.*, 2002b). A study by Shibuya et al. suggested that the induction apoptosis and/or necrosis in a melanoma (G361) cell line by the chemotherapeutic agents cisplatin or dacarbazine depended on the dose, which coincided with inverse expressions of Bax and Bcl-2 proteins (Shibuya *et al.*, 2003). Interestingly, it was found that the combination of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) with irradiation was highly effective against clonal HCT116 colorectal cancer cell lines independent of p53; however, cells deficient in BAX evaded elimination by this regimen (Ravi and Bedi, 2002). Therefore, Bax expression in our study of the melanoma cell line may play a role in its enhanced CPT and IR-mediated necrotic cell death but it is unlikely p53 is involved since these toxic agents failed to induce p53 expression. With this in mind, these findings may facilitate the development of regimens that modulate Bax expression for the treatment of cancer. The importance of this study demonstrates how initiating different modes of cell death can influence the responsiveness of tumour tissues to a variety of toxic agents and by manipulating these mechanisms one may overcome drug and radiation resistance in the clinical thereby minimizing normal tissue damage and maximizing tumour tissue death.

6.1.2 *General discussion on the assessment of whether the formation of radioresistant cells occurred by selection or adaptation to X-radiation treatment*

Further investigations with a human tumour colorectal cell line (HCT116), known to be CPT-sensitive, have shown that CPT can drastically potentiate cell killing when combined with fractionated doses of X-radiation, in comparison with the non-transformed fibroblast cell line (AG1522). Additional studies examining the effect of fractionated X-radiation on the acute radiation response of these cells in the presence of CPT produced data illustrating that HCT116 cells that had been previously treated with fractionated X-radiation alone were more resistant to single dose of X-radiation treatment than compared with cells that had not been treated with fractionated radiation.

This interesting discovery required further scrutiny and in order to study the mechanisms of radiosensitivity a cell model system had be employed that minimized the individual cellular cytogenetic differences to allow for isolation of radioresponsive genes, which was the aim of objective 3. Another rationale for acquiring this cell model system was that cells obtained directly from tumours are characteristically heterogeneous in nature and are composed of many different cell types in many different stages of differentiation (Aabo, 1996; Yu *et al.*, 2002). It is these heterogeneous radiation responses within a malignancy that often are the cause for local failure of radiotherapy (Brattain *et al.*, 1981; Leith *et al.*, 1982). However, cell lines have been generally accepted to being homogenous but they too may become heterogeneous over a number a cell culturing passages (Engelholm *et al.*, 1985; Gagos *et al.*, 1996). Therefore, the

selection of the colorectal cancer cell line for our study is founded on the fact that these cells provide an excellent model system for analyzing the roles of selection and genomic instability in tumours, because they are a relatively well-established cell line and because of the isolation of sister-clones derived from a parental cell population. The HCT116 cells also possess an increased intrinsic mutation rate, owing to defective mismatch repair (MMR) (Tomlinson and Bodmer, 1999) which may contribute to the production of cell clones with variable intrinsic radioresponses. Cytogenic studies, performed in our study, found the clones to be derived from the same cell line; free of possible foreign cell contamination and are of human origin. Our investigation clearly illustrates that well-established cell lines can contain heterogeneous phenotypes and that these differences existed independently of treatment since fractionated radiation generally did not alter radioresponsiveness of a number of cell clones. The significance of this study shows that cellular selection by a treatment can occur within a single cell line that can determine the radioresponsiveness of that cell population. Furthermore, this study can provide some insight of the possible mechanisms of somatic evolution that can lead to the generation of more resistant forms of cancer. The prevalence of radioresistant tumour cells by either selection or adaptation gives us some insight on predicting how a tumour may behave when given radio- and/or chemo-therapy and would direct the type of therapy that would be required to best treat that particular malignancy thus ultimately improving the diagnosis, treatment and prevention of cancer.

6.1.3 *General discussion on the isolation and comparison of sister clones with varying radioresponses, and non-overlapping drug sensitivities*

The main contribution of chapter 4 was the identification of clones with varying intrinsic radioresponses which have non-overlapping radiation and drug sensitivities. The importance of these findings could be in helping to develop a possible preclinical model system that would be useful for differentiating the genetic basis underlying the response of human tumours to these treatments. The most promising result from our findings is the identification of radiosensitive HCT116-CloneK, which displays resistance to a number of DNA damaging, cell cycle and alkylating agents. Interestingly, the works of Mattern et al., 1991, demonstrated that particular camptothecin resistant cell lines, characterized by a reduced topoisomerase-I content, are relatively more sensitive to IR than camptothecin-sensitive cells (Mattern *et al.*, 1991). Therefore, it would be of some interest to determine the topoisomerase-I status of HCT116-CloneK since it is the most camptothecin resistant and yet is the most radiosensitive of the parental HCT116 cells and the other cell clone progeny.

Few cell model systems show independent radiation and drug sensitivities and the isolation of HCT116-CloneK with non-overlapping drug and radiation responses provides an unique opportunity to potentially examine the signaling pathways involved in chemoresistance separate from radiation responses. Mechanisms of cross-resistance has been linked to higher than normal levels of free radical scavengers like glutathione and/or

thiol-containing proteins and/or by non-protein sulphydryl molecules (Richardson and Siemann, 1994). Alterations in DNA repair have also been implicated in multidrug and radiation resistance. For example, an increase in heterodimeric Ku autoantigen protein composed of 86-kDa (Ku80) and 70-kDa (Ku70) subunits has been suggested to be responsible for the phenotype of cross-resistance to IR and CDDP (Frit *et al.*, 1999). Decreased levels of the DNA topoisomerase I and II enzymes were also suggested to play a role in response to both DNA topoisomerase related chemotherapeutic agents and IR (Hoban *et al.*, 1992); a mechanism which may contribute to the radioresistance of Sk-Mel3 cells since topoisomerase I content was found in our laboratory to be relatively higher in the radiosensitive HT-144 human melanoma cell line (Ng *et al.*, 1998). Lastly, the management of apoptosis has also been held responsible for conferring cross-resistance to many other classes of drugs and IR (Johnstone *et al.*, 2002; Martin-Aragon *et al.*, 2000; Nicholson, 2000) and have been shown to share the same cell death pathways (Reinhold *et al.*, 2003). Therefore, cell model systems, such as HCT116-CloneK, with non-overlapping drug and radiation sensitivities allows for a better comprehension of the signaling pathways involved in either drug or radiation resistance can provide a significant therapeutic benefit since these therapies can potentially be targeted specifically to repress or inhibit pathways associated with either chemoresistance or radioresistance while minimizing detrimental affects to other vital cellular signals.

Our HCT116 clones also displayed similar UV but differing IR responses. A similar study demonstrated that human prostate cancer (DU145) cells were resistant to CPT and gamma-irradiation but were still quite responsive to UV irradiation (Reinhold *et*

al., 2003) which is not surprising since the types of DNA damage from UV radiations are known to initiate different modes of DNA repair. Analysis of specific genes associated with IR radiation- and UV-induced apoptosis in mouse thymic lymphoma (3SB) cell clones, also displaying resistance to IR but sensitivity to UV, showed separate radiation-specific p53-dependent and independent apoptosis pathways (Kawai *et al.*, 1998). Therefore, these clones as well as the ones generated in our laboratory may provide a useful tool to identifying the genes involved in the signaling pathways associated with either X-ray- or UV- mediated cell death.

There is still a great deal of speculation about the changes in genetic expression that are required to confer radioresistance in cells since radioresistance may involve a change in a number of radiation-associated and non-radiation-associated gene expressions. Another source of uncertainty in determining the genes that affect cell radiosensitivity is that the majority of studies are commonly performed in irradiated cells and fail to determine the genetic origin of their radioresponse, apart from the treatment (Achary *et al.*, 2000; Li *et al.*, 2001; Park *et al.*, 2002; Pearce *et al.*, 2001). For this reason, it was advantageous for us to do a simultaneous analysis of a large group of genes, in the non-irradiated clones, to give a better prediction of the genes required to confer a particular radiation response, while, gene expression analysis following XR treatment will only give insight on the effect of radiation on gene expression. Therefore, our hypothesis, was that basal gene expression in untreated cell clones could predict and determine the cellular response to radiation. As it is discussed in the next section, we identify a variety of intrinsic gene expressions that may play a role in determining how a

cell may response to X-radiation and that the radiation response of a tumour cell line may be dependant on the collective radiosensitivities of the individual cell clones that survive the treatment.

6.1.4 *General discussion on the genetic basis of radiosensitivity*

To complete the fourth objective, we sought to examine whether gene expression profiling of nonirradiated HCT116 clones of varying radiosensitivies could identify genes that are responsible for their X-radiation responses. Our findings in chapter 5, had identified that the majority of genes expressed in the analysis did not have previously known links to IR responses. The possible significance of this finding is that it identifies possible new genes or genetic pathways involved in modulating the radiation response that have been previously unidentified, potentially providing new venues which could then be utilized in designing new therapeutic regimes for the treatment of cancer.

One should note that just because a certain gene or a set of genes are involved in radiation protection in one cell type does not mean that these factors will have the same effects in another cell type. For example, some cell types show radioprotection through enhanced basal signaling by the proto-oncogene RAS. In most of these cells, RAS-mediated protection from IR is signaled by PI3K activation whereas in other cell types radioresistance is bestowed through the transcription factor, nuclear factor kappa B (NFKB) or MAPK signaling (Dent *et al.*, 2003). Microarray profiling of the HCT116 clones produced only one gene known to have a direct link with cellular IR

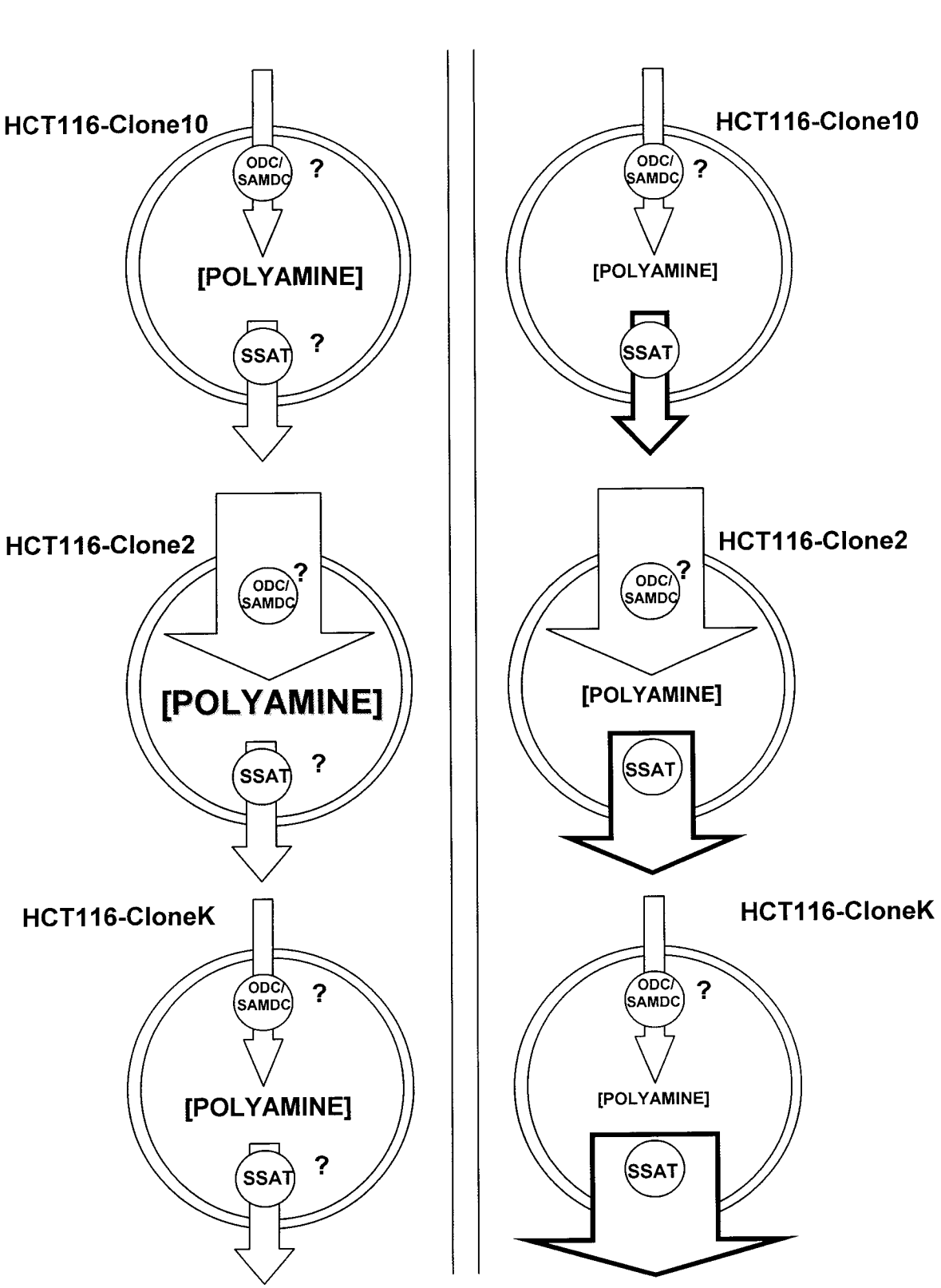
responsiveness in our investigation. Spermidine/Spermine N^1-Acetyltransferase (SSAT) had enhanced basal expression in the nonirradiated, radiosensitive HCT116-CloneK as compared to the HCT116 parental cells and the other HCT116 clones. SSAT is an enzyme that catabolizes polyamines that are known to protect against radiolytic attack of DNA from hydroxyl radicals produced by IR exposure (Sy *et al.*, 1999). Depletion of cellular polyamines has also been shown to impair DSB repair (Snyder, 1989; Snyder and Lachmann, 1989), inhibit cell proliferation and initiate cell death (Thomas and Thomas, 2001). Human melanoma (SK-MEL-28) cells treated with the polyamine analog N^1, N^{11}-diethylnorspermine (DENSPM) induced SSAT resulting in mitochondrial damage, an increase in oxidative products and apoptosis via and the caspase cascade (Chen *et al.*, 2001). A direct link between radiosensitivity and high levels of SSAT may have been established if non-stimulated SSAT protein levels of the three clones (10, 2, and K) were similar to the basal mRNA expression levels and/or increased in response to ionizing radiation. Regrettably, basal levels of SSAT proteins were undetectable in all clones with or without ionizing radiation. This is commonly seen in other reports and is often circumvented by the overexpression of SSAT gene or by the artificial induction of the enzyme by a polyamine analog such as DENSPM (Casero *et al.*, 1994; Murray-Stewart *et al.*, 2003). Since, overexpression of SSAT would not exploit clonal differences in SSAT levels, we had to artificially induce the SSAT enzyme in the clones using DENSPM. Artificial induction of the SSAT enzyme by DENSPM had the greatest effect in protein expression and enzymatic activity in radiosensitive HCT116-CloneK which reflected the higher basal gene expression of SSAT observed with this clone relative to the other sublines. This suggests the possible importance of SSAT gene expression for the IR

response in closely-related human colorectal (HCT116) clones which also provide a potential tool in predicting IR response. However, the inability of DENSPM to radiosensitize HCT116-CloneK, even though HCT116-CloneK polyamines levels had significantly decreased in the presence of DENSPM, shows that SSAT function may not be the only contributing factor in the radioresponse and that other mechanisms including regulators of polyamine synthesis and metabolism (i.e. ODC) should be examined along with SSAT, in order, to be used as a potential indicator of radioresponse.

Recently, an independent DNA microarray study has identified SSAT as a biomarker of resistance to the chemotherapeutic agent 5-fluorouracil (5-FU). In the breast cancer (MCF-7) cell line, SSAT mRNA was found to be induced 15-fold compared with control after a 48 h treatment with 10 uM 5-FU. Cytotoxicity of 5-FU treatment has been shown to involve p53 and SSAT has been suggested to interact with p53 via three putative p53-binding sites. Treatment with 5-FU of MCF-7 cancer cells devoid of p53, induced SSAT mRNA only 2-fold versus 15-fold in cells containing wild-type p53. Furthermore, basal expression levels of SSAT mRNA were increased 2-3-fold in a 5-FU-resistant colorectal cancer (H630-R10) cell line (Maxwell *et al.*, 2003). Chapter 4 also reveals that the radiosensitive HCT116-CloneK was the most resistant to four drugs relevant to chemotherapy which demonstrates that SSAT may have a putative role in not only radiation resistance but also chemoresistance. These studies clearly demonstrate the potential of DNA microarrays to identify novel genes involved in mediating the response of tumour cells to both radiotherapy and chemotherapy.

Even though, DENSPM was unable to radiosensitize HCT116-CloneK, the combined modality treatment of DENSPM and XR did reduce clonogenic survival and significantly enhanced radiosensitization of HCT116-Clone10, HCT116 parental cell line, and, in particular, the radioresistant HCT116-Clone2. Enhanced cell death in these clones correlated with the increased SSAT activity and the depletion of intracellular radioprotective polyamines. These results suggest that artificial induction of SSAT enzyme activity and, more importantly, the depletion of intracellular polyamine levels of both spermine and spermidine by DENSPM, may play a role in determining the radioresponse of particular cell model systems. Since polyamines may be the determining factor in radiosensitivity then it is logical to suggest that other polyamine regulatory mechanisms other than SSAT may be involved. For example, the radioresistant HCT116-Clone2 contains significantly higher levels of polyamines than the other cell clones and yet contains a highly inducible SSAT enzyme. This may be a result of a higher level of orithine decarboxylase (ODC) or S-adenosylmethionine (SAMDC) activity or expression, both of which are involved in the synthesis of polyamines, (See figure 1) and the induction of SSAT by DENSPM may export or degrade the intracellular polyamines faster than it is synthesized thereby exposing potential radiolytic attack sites on DNA that is normally inaccessible because of bound polyamines to these sites. In the case of HCT116-CloneK the lack of radiosensization may be a result of the already low production of polyamines and the induction of SSAT by DENSPM was unable to further lower polyamine levels to any appreciable level thereby having very little affect on survival (See figure 1).

Figure 1. A hypothetical model illustrating polyamine regulation in the cell clones with and without DENSPM.


- DENSPM
+ DENSPM
HCT116-Clone10
ODC/ SAMDC
?
[POLYAMINE]
SSAT
?
HCT116-Clone10
ODC/ SAMDC
?
[POLYAMINE]
SSAT
HCT116-Clone2
ODC/ SAMDC
?
[POLYAMINE]
SSAT
?
HCT116-Clone2
ODC/ SAMDC
?
[POLYAMINE]
SSAT
HCT116-CloneK
ODC/ SAMDC
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HCT116-CloneK
ODC/ SAMDC
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[POLYAMINE]
SSAT

It is also conceivable that other mechanisms, other than those associated with polyamines, which may include the other genes identified by the microarray analysis, that may explain the radiosensitivity observed with HCT116-CloneK. Nevertheless, the data clearly provides evidence that a combinational regimen of IR treatment with drugs such as DENSPM which can deplete polyamine levels may be a useful strategy for combating drug and radiation resistance in the clinic. Only a small number of studies have investigated this prospect including a study by Chen *et al.* which demonstrated that the cytotoxic polyamine analog N1,N14-bis(ethyl)homospermine (BE-4-4-4), increased IR cytotoxicity in the human glioma tumour (U-251 MG) cell line (Chen *et al.*, 1994). Another report by Bock *et al.*,1999 showed that exposure of head and neck squamous cell carcinomas to alpha-difluoromethylornithine (DFMO) depleted intracellular putrescine and spermidine levels greater than 5-fold and prevented cell proliferation, without the expression of cell death, as measured by a clonogenic assay. Unfortunately, the combination DFMO and single-dose radiation between 0 and 10 Gy did not influence the survival response in these cells. However, treatment of polyamine-depleted cells with the chemotherapeutic agent, staurosporine amplified its normal cytotoxicity by apoptotic cell death as compared to the controls. This suggested possible new therapeutic strategies combined with radiation therapy in head and neck cancer patients (Bock *et al.*, 1999). Lastly, the potential therapeutic efficacy of the lead spermine analogue 1,12-diaziridinyl-4, 9-diazadodecane (BIS) was tested in advanced prostate cancer with *in vitro* exposure to BIS in conjunction with gamma-irradiation resulting in a significant increase in the number of apoptotic cells as well as a decrease of clonogenic survival. *In vivo* exposure to BIS with irradiation also demonstrated a significant antitumour effect providing

evidence for the clinical implications of the polyamine analogue for the optimization of the therapeutic efficacy of radiation in patients with advanced prostate cancer (Eiseman *et al.*, 1998). Therefore, the polyamine analogs or drugs that modulate polyamine levels can potentially increase the radiosensitivity of cancer cells by lowering the apoptotic threshold to radiation and can thereby be a potential therapeutic agent for the treatment of specific types of cancers.

6.1.5 *Ongoing and future experiments on the characterization of radiosensitivity*

In this dissertation, we give evidence that SSAT expression can be a predictor of radiation response; however, this does not address whether SSAT has a direct role in radioresponsiveness. For this to be determined, the SSAT gene can be artificially enhanced by inserting the gene in an expression vector and transfected, either stably or transiently for the duration of IR exposure, into several different cell lines with a range of radioresponsiness. The artificial expression of this gene and its translation, as determined by Q-PCR and immunoblotting, as well as its effects on polyamine levels should provide evidence for its role in conferring radiosensitivity. Similarly, cellular expression of an antisense SSAT gene should reduce intrinsic basal levels of SSAT, increasing intracellular polyamine levels and enhancing radioresistance, to a greater extent, in cells that normally express high levels of SSAT.

One cannot ignore the relative importance of cell cycle distribution on radiosensitivity, and for this reason the observed differences between the cell clones

could be, to some extent, a result of variations in the stage or duration of the cell cycle phases (Blakely *et al.*, 1989; Wilson, 2004). However, fractionated X-radiation therapy, known to cause shifts to more radiosensitive phases of the cell cycle (Ngo *et al.*, 1988; Pawlik and Keyomarsi, 2004; van Oostrum *et al.*, 1990), did not alter sensitivity of clones 2 and 10. Nevertheless, it may still be a significant factor in causing induced radioresistance in clone K and 4, since the inducible resistance response in clone 4 was observed only following fractionation and not when given a single priming dose prior to a challenge dose. So, the fractionated treatment may cause the selection of more resistant s-phase cells amongst asynchronous cycling cells within the clone parental population. To confirm this hypothesis, additional experiments are required, using flow cytometry, to assess cell cycle distribution in the clones following exposure to single and fractionated doses of X-radiation.

Similarly, the DNA repair capability of a cell can also be a factor in determining the extent of radioresistance (Rothkamm *et al.*, 2003). Even though no obvious changes in expression of DNA repair genes or genes associated with repair were observed in the DNA microarray results of chapter 5, future experiments are still essential to assess the DNA repair capacity of the clones to both single and double strands breaks, using a sensitive technique that detects DNA damage and repair at the level of single cells, known as single cell gel assay or the comet assay (Olive *et al.*, 1990).

As mentioned previously in chapter 5, SSAT was amongst a number of other genes that were found to be either upregulated or downregulated by more than two fold

when considering the gene expression profiles of both HCT116-Clone2 and HCT116-CloneK together. These additional genes include adipose specific most abundant gene 2 (APM2), vitamin D 24-hydroxylase (CYP24A1) and XRRA1. XRRA1 was originally identified from our microarray investigation as a downregulated enhanced sequence tag sequence (EST), accession no. R40588, in the IR-resistant cell clone HCT116-Clone2. This single novel gene was assembled using this EST sequence as well as 30 other overlapping ESTs sequences and was termed by our laboratory as X-ray radiation resistance associated 1 (XRRA1) (GenBank BK000541). This was the first report on the molecular cloning, genomic characterization and over-expression of the XRRA1 gene and its role in the response of human tumour cells to IR (Mesak *et al.*, 2003). Investigation of XRRA1 gene expression had shown it to be presented predominantly in the testis of both human and macaque with its expression being found in most tumour cell types. The XRRA1 protein was also found to be highly conserved in many different species including human. Q-PCR analysis demonstrated an association with XRAA1 and IR as exposure to 4 Gy of IR was found to immediately increase expression of XRAA1 in the radioresistant HCT116-Clone2, but this decreased soon after IR exposure. Conversely, gene expression of XRAA1 of the other two clones decreased following IR, but this recovered to basal levels only in the radiosensitive clone HCT116-CloneK 24 hours later. Interestingly, over-expression of XRRA1 in COS-7 cells was found to lack the non-homologous end joining (NHEJ) DNA repair molecule Ku86 which, along with Ku70, are likely the major players in DSB rejoining and repair following DNA damage by IR (Jeggo, 1998), and possibly partly responsible for radioresistance. Additional studies are needed to confirm these speculations but the results so far suggest that

XRRA1 may be involved in the early response of cells to IR, and that XRRA1 may affect the radioresponses of HCT116 clones through signaling of DNA damage or by mediation of cell death. (Mesak *et al.*, 2003).

Additional experiments are underway to examine the roles of the additional highly expressed genes, APM2 and CYP24A1. *APM2* is a novel gene first identified in adipose tissue possessing unknown function. By comparison, the human adipocyte-specific APM-1 gene encodes for a adipose secretory protein that has been suggested to play a role in the pathogenesis of obesity (Schaffler *et al.*, 1998; Schaffler *et al.*, 1999); however, its role in either radioresistance or chemoresistance is not apparent and warrants further investigation. The enzyme product of CYP24A1 (cytochrome P450, subfamily XXIV, vitamin D 24-hydroxylase) is known to maintain calcium homeostasis and catalyzes the NADPH-dependent 24-hydroxylation of 25-hydroxyvitamin D(3), the biological active metabolite of vitamin D. We show CYP24 to be downregulated in the radiosensitive HCT116-CloneK which is of particular interest since downregulation of CYP24A1 results in higher levels of bioactive vitamin D(3) (Schuster *et al.*, 2003). This has been shown to induce apoptosis in MCF-7 cells by initiating mitochondrial dysfunction, mediated through the translocation of Bax to the mitochondria, resulting in the release of cytochrome c and the subsequent production of reactive oxygen species (ROS). For this reason, the usage of vitamin D(3) related compounds has been suggested as an alternative approach for treating human breast cancer (Narvaez *et al.*, 2003), with the initiation of apoptosis and the generation of ROS possibly helping to radiosensitize tumours to IR.

Array analysis on clones following fractionated IR treatment would also be of great significance since modulation of gene expression by irradiation could play a role in tumour radiosensitivity. In order to investigate this area of interest, our laboratory has also generated clones with varying radiosensitivities (i.e. radioresistant 7B, radiosensitive 3A, and normal 5) selected from HCT116 parental cells following irradiation with ten 2 Gy fractions that are currently awaiting further investigation.

6.1.6 *Conclusions*

In conclusion, the data suggests that the radioresponse of a tumour cell line may be determined by the presence of the relatively radioresistant sublines within the parental population. Moreover, the clones, selected from the non-irradiated parental cell line, contained distinctive basal gene expressions that could potentially predict their individual radioresponses. From amongst these significant changes in genetic expression, some of which were not previously thought to be associated with the radiation response, we have identified SSAT as a possible predictor of the cellular response to IR and as a potential modulator of radiosensitivity which could provide some additional clues to the understanding of radioresistance and thus encourage a treatment for overcoming radioresistance in cancer therapy. For example, various preclinical studies using polyamine derivatives have been shown to have encouraging results in reducing tumour progression (Bernacki *et al.*, 1995; Davidson *et al.*, 1999; Hahm *et al.*, 2001; Hahm *et al.*, 2002; Porter *et al.*, 1993). In addition, more current studies are investigating innovative

therapies involving the administration of a novel set of third generation polyamine analogs, which have already been demonstrated to have even more promising results in the treatment and prevention of a number of cancers (Valasinas *et al.*, 2001; Wolff *et al.*, 2003). However, the majority of these studies that examine the antitumour affects of these analogs are normally given either alone or in combination with other cytotoxic drugs in the absence of IR. In contrast, we have demonstrated in a number of the colorectal cancer cell clones that the toxicity of IR can be enhanced when combined with the first generation polyamine analog N^1, N^{11}-diethylnorspermine (DENSPM) thereby providing a rationale for the administration of polyamine analogs to improve conventional radiotherapy through the radiosensitization the relatively more radioresistant tumour cells.

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6.3 ADDENDUM

6.3.1 *Addendum to Chapter Two*

1. **Q. What does the statement "... can potentially enhance the response of non-resectable primary melanomas to treatment . . ." mean? Is it a typographical error?** See Chapter 2, page 68, paragraph 1.

   Answer.

   It means that the combination of chemotherapy with radiation could be more effective than giving radiation alone to melanomas that can't be removed surgically.

2. **Q. The term "adjunct" to radiation is imprecise. I would change suggest you state that camptothecin is a useful radiosensitizer. Otherwise, please define the word "adjunct".** See Chapter 2, page 69, paragraph 2.

   Answer.

   Adjunct – "Something joined or added to another thing, but not essentially a part of it." Source: Websters Dictionary (Mar 1, 1998). The citations stated show that camptothecin given in combination with radiation can be useful since the drug given before, during or after radiation exposure can enhance lethal effects. (Boothman, D.A., Wang, M., Schea, R., Burrows, H.L., Strickfaden, S., Owens, J.K. (1992) Posttreatment exposure to camptothecin enhances the lethal effects of X-rays on radioresistant human malignant melanoma cells. Int. J. Rad. Oncol. Biol. Phys. 24: 938-948) (Chen, A.Y., Okunieff, P., Pommier, Y., Mitchell, J.B. (1997) Mammalian DNA topoisomerase I mediates the enhancement of radiation cytotoxicity by camptothecin derivatives. Cancer Res. 57: 1529-1536) (Musk, S.R.R., Steel, G.G. (1990) The inhibition of cellular recovery in human tumour cells by inhibitors of topoisomerase. Brit. J. Cancer 62: 364-367) (Zanier, R., De Salvia, R., Fiore, M., Degrassi, F. (1996) Topoisomerase I activity and cellular response to radiation in Chinese hamster cells. *Int. J. Rad. Biol.* 70: 251-259)

3. **Q. I am not sure what you mean by this statement. Are you saying that the hypothesis is that both apoptotic and necrotic cell deaths are important, as opposed to only one of these 2 methods of cell death being important? Are you implying that agents lethal to most cancer cells involve one or the other mechanism, only?** See Chapter 2, page 69, paragraph 2.

   Answer.

It has been shown that not only can apoptosis be initiated as an intrinsic death program but also necrosis can also be triggered intrinsically and may not only be a form of chaotic cell death. Necrosis has been demonstrated to be involved in embryonic development and can be induced by tumour necrosis factor (TNF) in certain cells under particular conditions (Chautan, M., Chazal, G., Cecconi, F., Gruss, P., Golstein, P. (1999) Interdigital cell death can occur through a necrotic and caspase-independent pathway. *Curr Biol.* 9(17):967-70.)(Vercammen, D., Brouckaert, G., Denecker, G., Van de Craen, M., Declercq, W., Fiers, W., Vandenabeele, P. (1998) Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. *J Exp Med.* 188(5):919-30.)(Vercammen, D., Beyaert, R., Denecker, G., Goossens, V., Van Loo, G., Declercq, W., Grooten, J., Fiers, W., Vandenabeele, P. (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumour necrosis factor. *J Exp Med.* 187(9):1477-85.). The mode of cell death can be also be altered, using signaling pathway inhibitors, leading to the dominance of one of these cell death mechanisms (Kalai, M., Van Loo, G., Vanden Berghe, T., Meeus, A., Burm, W., Saelens, X., Vandenabeele, P. (2002) Tipping the balance between necrosis and apoptosis in human and murine cells treated with interferon and dsRNA. *Cell Death Differ.* 9(9):981-94.). This is important since the generation of an appropriate immune response following apoptosis or necrosis can be essential for either antitumour or antiviral responses. One factor for the involvement of the immune system is that apoptotic cells are rapidly removed *in vivo* without causing a significant immune response whereas necrotic cells continue to synthesize protein to the point of lysis resulting in an inflammation response to the released intact cytosolic proteins (Rathmell, J.C., Thompson, C.B. (1999) The central effectors of cell death in the immune system. *Annu Rev Immunol.*17:781-828.) (Saelens, X., Festjens, N., Parthoens, E., Vanoverberghe, I., Kalai, M., van Kuppeveld, F., Vandenabeele, P. (2005) Protein synthesis persists during necrotic cell death. *J Cell Biol.* 168(4):545-51.). Our paper was to determine the importance of the contribution of either apoptosis or necrosis or the combination of both in cancer cells treated with toxic doses of camptothecin. Different cancer cells may respond differently where one death mechanism may be prevalent than another or both mechanisms could be as equally as present. Knowledge about the mechanisms by which these agents influence these death pathways in neoplastic diseases may suggest strategies for more effective and less toxic therapies for patients suffering from these malignancies. (Kolenko, V., Uzzo, R.G., Bukowski, R., Bander, N.H., Novick, A.C., His, E.D., Finke, J.H. (1999) Dead or dying: necrosis versus apoptosis in caspase-deficient human renal cell carcinoma. *Cancer Res.*, 59(12):2838-42.) (Gervasoni, J.E. Jr., Hindenburg, A.A., Vezeridis, M.P., Schulze, S., Wanebo, H.J., Mehta, S. (2004) An effective in vitro antitumour response against human pancreatic carcinoma with paclitaxel and daunorubicin by induction of both necrosis and apoptosis. *Anticancer Res.*, 24(5A):2617-26.) (Delhalle, S., Duvoix, A., Schnekenburger, M., Morceau, F., Dicato, M., Diederich, M. (2003) An introduction to the molecular mechanisms of apoptosis. *Ann N Y Acad Sci.*, 1010:1-8.)

4. **Q. Please cite references to this statement. Are these references the some ones as those you cite for the next statement?** See Chapter 2, page 69, paragraph 3.

Answer.

The reference was not cited. The morphology and differentiated functions of melanocytes are known to be influenced by environmental factors such as ultraviolet and ionizing radiations. However, it has been shown that the transient radiation response of mouse melanocytes over the dose range 0.9 to 10 Gy was variable. (Vegesna, V., Withers, H.R., Taylor, J.M., (1987) The effect on depigmentation after multifractionated irradiation of mouse resting hair follicles. *Radiat Res.*, 111(3):464-73.). It is probably for this reason that melanocytes are but are not often used as a standard for examining late tissue effects in patients exposed to ionizing radiation. This can be determined by examining the *in vitro* radiosensitivity of skin fibroblasts by way of the clonogenic assay from a simple punch skin biopsy, which has been shown to correlate with late complications in normal tissues following radiotherapy. (Alsbeih, G., Malone, S., Lochrin, C., Girard, A., Fertil, B., Raaphorst, G.P. (2000) Correlation between normal tissue complications and in vitro radiosensitivity of skin fibroblasts derived from radiotherapy patients treated for variety of tumours. *Int J Radiat Oncol Biol Phys.*, 46(1):143-52.)

5. **Q. Please mention the city, state/country of the supplier Boehringer Mannheim.** See Chapter 2, page 70, paragraph 1.

Answer.

Boehringer Mannheim, Mannheim, Germany

6. **Q. Although DMSO is a chemical known to most scientists by the abbreviation, I would in a thesis put the full name of the chemical (the first time you mention it in the thesis). Should you mention the supplier's name of DMSO? Should you mention why the CPT was dissolved in DMSO, rather than the more usual aqueous solvents (you might mention the lack of solubility of CPT in these other solvents).** See Chapter 2, page 70, paragraph 2.

Answer.

The full chemical name of DMSO is dimethyl sulfoxide (Sigma Chemical Co, St. Louis, MO. Cat. #: D-5879). DMSO is a solvent known to enhance drug solubility (Kawakami K, Miyoshi K, Ida Y. (2004) Solubilization behavior of poorly soluble drugs with combined use of Gelucire 44/14 and cosolvent. *J Pharm Sci.*, 93(6):1471-9.). Yes, camptothecin is relatively insoluble in aqueous solutions but is more soluble in the lipophilic solvent DMSO (I found the maximum concentration to be 20mM) and can then be further

dissolved in media. (Pizzolato JF, Saltz LB. (2003) The camptothecins. Lancet. 361(9376):2235-42.) (Dai, J.R., Hallock, Y.F., Cardellina, II J.H., Boyd, M.R., (1999) 20-O-beta-glucopyranosyl camptothecin from mostuea brunonis: A potential camptothecin pro-drug with improved solubility. J Nat Prod. 62(10):1427-9.) Also, see page 34 of INTRODUCTION **under the heading** 1.5 RADIORESISTANCE AND CHEMOTHERAPY

7. **Q. You mention the dose rate of 150 cGy/min. What is the duration of the radiation treatment?** See Chapter 2, page 70, paragraph 2.

Answer.

For the majority of experiments, the duration for 2 Gy @ a dose rate of 1.5 Gy/minute is 1 minute and 20 seconds. + 3.2 seconds for an initial warm-up time. Therefore, the total time to set the timer is 1 min and 23 seconds for a dose of 2 Gy.

More recent experiments were given 1 minute and 7 seconds for a 2 Gy dose since the dose rate had changed to 1.892 Gy/minute (i.e. 1 min and 3.4 seconds + an additional 3.2 sec = 1 min and ~ 7 sec) after the X-ray tube was replaced.

7. **Q. You should also mention if DMSO is itself toxic to the cells, compared to a control aqueous solvent? If it is, one would wonder that any effects of CPT on the cells may actually be due to the interation of DMSO with CPT on the cells.** See Chapter 2, page 71, paragraph 2.

Answer.

Dimethyl sulfoxide (DMSO) can have effects on the cell in high enough concentrations but is known to have extraordinary low toxicity and is used to prevent ice crystals from forming in cells when placed for cryo-preservation. (Walter, Z., Szostek, M., Weglarska, D., Raguszewska, D., Jablonski, M., Lorenz, F., Skotnicki, A.B. (1999) Methods for freezing, thawing and viability estimation of hemopoietic stem cells. *Przegl Lek.*, 56 Suppl 1:34-9). DMSO properties include being an anti-inflammatory agent and free radical scavenger, and was shown to protect against acute bleomycin-induced pulmonary fibrosis in the rat (Haschek WM, Baer KE, Rutherford JE. (1989) Effects of dimethyl sulfoxide (DMSO) on pulmonary fibrosis in rats and mice. *Toxicology.* 54(2):197-205.) In high enough concentrations DMSO has been documented to induce either a non-immunological immediate contact urticaria (an allergic reaction characterized by pale or reddened irregular, elevated patches) or an irritant reaction when applied to the skin. (Sjogren, F., Anderson, C. (2000) The spectrum of inflammatory cell response to dimethyl sulfoxide. *Contact Dermatitis.*, 42(4):216-21). Additionally, DMSO is also a well-known differentiation inducer in several myeloid cells, and can induce a reversible G_1 arrest in many cell lines (Fiore, M., Zanier, R. and Degrassi, F. Reversible G_1 arrest by dimethyl sulfoxide as a new method to

synchronize Chinese hamster cells. *Mutagenesis*, Vol. 17, No. 5, 419-424).

Only very small volumes of DMSO were used in the studies performed within this dissertation (i.e. max 9.6 ul in 3 ml media) and a vehicle control containing DMSO alone was always used to normalize these possible effects.

8'. Q. **The medium was changed to what? To non-CPT containing DMSO?** See Chapter 2, page 70, paragraph 3.

Answer.

Yes.

8. Q. **What is the term "relative colony forming curves" (this and future figures) mean? In the text, what do you mean by the term "small"? Please quantitate, and also indicate if there was a statistically significant difference, or not.** See Chapter 2, page 73, paragraph 1.

Answer.

The term "relative colony forming curves" simply means the same as saying the surviving fraction curve.

The clonogenic survival for both cell lines at 2uM CPT was stated to be "small" meaning that at this dose the amount of survival was similar and insignificantly different since the error bars overlapped.

9. Q. **I believe, if 1 am not mistaken, that the abbreviation "RCF" is being used for the first time. Please state the full term, when you mention it for the first time.** See Chapter 2, page 73, paragraph 2.

Answer.

The full term was noted on See Chapter 2, page 75, paragraph 1.

10. Q. **In this paragraph (pages 73/74), you talk about differences and similarities, without mentioning if this is a subjective impression, or whether you used statistics (i.e. what does "significant decrease" mean?). You imply, that similar loss of AG1522 cells was observed to both concentrations of CPT but you do not explicitly state that. Even if you were to state it, I would recommend that you back up that (or a contrary) statement with statistics - as the curves, at the later time points, taking account the error bars, seem to be quite different.** See Chapter 2, page 76, paragraph 1

Answer.

This was based on the observation that 10-40 % of the melanoma cells remained attached after 1h of CPT treatment versus 90 % of the SK-Mel3 cells not treated with CPT. Whereas, the maximum of 10 % of the AG1522 cells treated with 1 h CPT was lost to the media compared to the non-treated cells. Based on the statistical students t-test both cell lines after 48 h showed significant decreases in attached cell number when treated with either the low or high dose of CPT versus the vehicle control $p<0.05$.

11. **Q. A better way to express the statement is to say that the number of cells remained constant/stable, with the exposure of 2 uM CPT.**

The next statement, uncaused a substantial reduction to fewer than the initial number..." is not very precise - are you stating that there was a substantial reduction in the number of cells, or are you stating that any reduction to fewer than the initial number is significant? You may wish to give a concrete number, in the sentence. See Chapter 2, page 76, paragraph 2.

Answer.

Yes, I meant to say that there was substantial reduction in the number of cells from 100% of the seeding density to 5-10%.

12. **Q. I am confused by the statement "both these doses were cytotoxic, not cytostatic..." If the cell number remains more or less constant, as with 2 uM CPT, that could mean that the death of cells – resulting in a relative plateau in the number of cells, as observed in Figure 4 – I thought that this would still constitute a cytostatic, but not a cytotoxic, effect?**

Answer.

I disagree, figure 3a shows that the percentage of attached cells in relation to total number of cells which include both the attached and floating fraction. It is clear that the percentage of number of floating cells after 48 h, with either dose of CPT, surpasses the number of attached cells. However, in figure 4, the measured attached cells are in relation to the original seeding density and does not give an indication of cell detachment and death. Based on this one can conclude that rapid cell growth is occurring in the Sk-Mel3 but the cells quickly become detached only replacing what they lost hence a cytotoxic effect. However, in the case of AG1522 cells the majority of these cells remain attached and do not come off and do not increase in cell number thus demonstrating a cytostatic effect.

13. **Q. Did you look at the percentage of apoptotic vs necrotic cell death, separately in the attached versus floating cells?**

Answer.

Yes.

14. **Q. Please state the actual median survival, in months, of metastatic melanoma - that is extensively documented.** See Chapter 2, page 82, paragraph 1.

Answer.

It was shown that data collected from 444 patients with metastatic melanoma between January 1978 through to December 1997 had a median survival time of 7 months, 17 months following curative resection, and 6 months after incomplete resection. (Meyer T, Merkel S, Goehl J, Hohenberger W. (2000) Surgical therapy for distant metastases of malignant melanoma. *Cancer.* 89(9):1983-91.)

Also, the patient survival is dependent on the location of the first metastasis, the resectability of the metastases and the aggressiveness of the metastases (Lee, S.M., Betticher, D.C., Thatcher, N. (1995) Melanoma: chemotherapy. *British Medical Bulletin,* 51(3):609-30).

For example, patients with distant skin, subcutaneous or nodal metastases only (M1a) have a median survival of 12.8 months; patients with lung metastases (M1b) have an 11.8-month median survival; and patients with visceral metastases or elevation of lactate dehydrogenase (LDH) (M1c) have a 7.8-month median survival." (Ernstoff, M.S. (2004) Therapeutic Strategies for the Treatment of Metastatic Melanoma – What is New is Old. *Business Briefing: US Oncology Review.*)

In another study, 102 patients with malignant melanoma who had distant metastases surgically resected and were judged to be clinically free of disease (M. D. Anderson Stage IVA melanoma) were studied. They determined that the median survival for all the patients from time of diagnosis of stage IVA disease to be 18 months. Interestingly, resection of the metastases did not improve survival, being approximately the same for the brain (15 months), lung (16 months), intraabdominal (18 months), and skin and/or lymph nodes (23 months). (Feun, L.G., Gutterman, J., Burgess, M.A., Hersh, E.M., Mavligit, G., McBride, C.M., Benjamin, R.S., Richman, S.P., Murphy, W.K., Bodey, G.P., Brown, B.W., Mountain, C.F., Leavens, M.E., Freireich, E.J. (1982) The natural history of resectable metastatic melanoma (Stage IVA melanoma). *Cancer.* 50(8):1656-63.)

15. **Q. I am not sure that the statement (with citation 1) is precise - radiation is indeed given in locoregional management of melanomas - the response of melanomas is a function of dose of radiation, volume of radiation (which depends upon volume of tumour) - I believe these are also relevant clinical**

factors, in addition to the fact that some patients have more radiosensitive melanomas than other patients. See Chapter 2, page 82, paragraph 1.

Answer.

Yes, I agree. The response of tumour tissue to radiation is dependant on a number of factors including the progression and severity of the patient's disease (whether they can tolerate the addition radiation therapy), the location of the tumour, tumour volume, oxygen availability, radiation dose per fraction, and the total dose. However, this particular statement focuses on a number of cases that some melanomas are relativity more radioresistant and may require additional agents (i.e. chemotherapy, hyperthermia, cytokines) for palliation and tumour remission with prolongation of survival. (Geara, F.B., Ang, K.K. (1996) Radiation therapy for malignant melanoma. *Surg Clin North Am.*, 76(6):1383-98.) (McWilliams, R.R., Brown, P.D., Buckner, J.C., Link, M.J., Markovic, S.N. (2003) Treatment of brain metastases from melanoma. *Mayo Clin Proc.*, (12):1529-36.) (Gupta, S., Kanodia, A.K. (2002) Biological response modifiers in cancer therapy. *Natl Med J India.*, 15(4):202-7.) (Iqbal, M., Marshall, E., Green, J.A. (2000) Ten-year survival in advanced malignant melanoma following treatment with interferon and vindesine. *Ann Oncol.* 11(4):483-5.)

16. **Q. Earlier in the paragraph (page 85), you talk about necrosis secondary to apoptosis. If a significant portion of your necrotic cells first underwent apoptosis, then would your concluding statements (about relative numbers of apoptosis and necrosis) would still be accurate, as stated by you in these sentences in the middle of the paragraph.**

It is possible, that even though apoptosis and necrosis were low in AG1522, that most of the necrotic cells did not undergo apoptosis first?

In terms of the Sk-Me1-3, who is to say that in the earlier time points, most of the necrotic cells first underwent apoptosis – i.e. maybe the time frame for a cell to undergo secondary necrosis, after it has already undergone apoptosis, is very minimal (maybe almost instantaneous?).

Do you have any data, either yours, or published, about the time frame of apoptosis, relative to primary necrosis, relative to secondary necrosis - in a given cell line, exposed to a given toxic agent, at various concentrations? See Chapter 2, page 85, paragraph 1.

Answer.

It is difficult to ascertain with absolute uncertainty the fraction of necrotic cells that first underwent apoptosis versus the necrotic death that is independent of apoptosis. The hypothesizes were constructed based on the observation of the

amount of necrotic death in relation to the amount of apoptotic death. It is possible that the AG1522 cells did undergo necrosis in the absence of apoptosis. This is most likely caused by mitotic-linked cell death in which these cells manifest a number chromosomal aberrations and transmit these aberrations to the replicated cells during cell division continuing to form colonies. However, these daughter cells containing these aberrations do not die (by either apoptosis or necrosis) over several generations but will eventually form large multinucleated cells that continue to live for several hours to days and finally die by way of (secondary) necrosis or apoptosis. (Forrester, H.B., Albright, N., Ling, C.C., Dewey, W.C. (2000) Computerized video time-lapse analysis of apoptosis of REC:Myc cells X-irradiated in different phases of the cell cycle. *Radiat Res.* 154(6):625-39.) (Yanagihara, K., Nii, M., Numoto, M., Kamiya, K., Tauchi, H., Sawada, S., Seito, T. (1995) Radiation-induced apoptotic cell death in human gastric epithelial tumour cells; correlation between mitotic death and apoptosis. *Int J Radiat Biol.* 67(6):677-85.) However, even though I agree that a portion of necrotic cells (in the absence of apoptosis) may account for some of the loss of clonogenic survival (less than 20 %) with 25 uM CPT. I believe that the low levels of apoptosis and necrosis along with the consistently higher portion of apoptotic cells in relation to the necrotic cells during the 120h time period suggests that necrotic cells that did not undergo apoptosis first may be insignificant.

In one study, serum was removed from the media surrounding AKR-2B fibroblasts. Time-lapse video microscopy was used to measure both the dynamic structural changes and to measure the time span of individual cells. Cell death appeared to occur after 90 minutes and reached a plateau after six hours with 40-50% of the cells being viable for at least 48 hours. Membrane blebbing occurred within 45 minutes and continued for 140 minutes with no further structural change. After 140 minutes membrane vesicles are lost and the nucleus becomes prominent and remains this way for an additional 12 hours. Necrotic cells were characterized by high propidium iodide fluorescence and a low rhodamine 123 fluorescence and the appearance of these necrotic cells occurred after 4 hours of serum starvation. (Simm, A., Bertsch, G., Frank, H., Zimmermann, U., Hoppe, J. (1997) Cell death of AKR-2B fibroblasts after serum removal: a process between apoptosis and necrosis. *J Cell Sci.*, 110:819-28.) However, several reports claim that time lines of cell death are different depending on the cell type. (Kaufmann SH. (1996) Proteolytic cleavage during chemotherapy-induced apoptosis. *Mol Med Today.* 2(7):298-303.)(Cohen JJ. (1993) Apoptosis: the physiologic pathway of cell death. *Immunol Today.* 14(3):126-30.)

I agree, secondary necrosis following or during apoptosis can be instantaneous and therefore could be measured within hours after drug treatment. For this reason, a fraction of the necrotic Sk-Mel3 cells at the earlier time points could have previously underwent apoptosis. Keep in mind though that primary necrosis could occur at any time especially immediately after or during drug or radiation treatment in that the treatment physically comprised the cell to the point of loss of

cell function and the membrane being comprised (Bradbury DA, Simmons TD, Slater KJ, Crouch SP. (2000) Measurement of the ADP:ATP ratio in human leukaemic cell lines can be used as an indicator of cell viability, necrosis and apoptosis. *J Immunol Methods.* 240(1-2):79-92.) (Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M.A., Lassota, P., Traganos, F. (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry.*13(8):795-808.). However, the death associated with the combined treatment may be partly a result of secondary necrosis but is not a major contributor since a significant increase in necrosis occurred with no change in the apoptotic index. Depending on how the secondary necrosis is initiated either following apoptosis or caused by mitotic-linked death the process could occur after several hours or days. (Dewey, W.C., Ling, C.C., Meyn, R.E. (1995) Radiation-induced apoptosis: relevance to radiotherapy. *Int J Radiat Oncol Biol Phys.* 33(4):781-96.)

17. **Q. The 2 statements related to unpublished data - does this data have any significance to the data presented in this chapter of your thesis.** See Chapter 6.3, page 243.

Answer.

It does have significance since functional wild type p53 is often responsible for initiation of apoptotic death and it is known that 50% of most tumour types possess a mutant form of p53 and are often less sensitive to the effects of drug and radiation treatment. (Soussi, T., Kato, S., Levy, P.P., Ishioka, C. (2005) Reassessment of the TP53 mutation database in human disease by data mining with a library of TP53 missense mutations. Hum Mutat. 25(1):6-17.) (O'Connor, P.M., Jackman, J., Jondle, D., Bhatia, K., Magrath, I., Kohn, K.W. (1993) Role of the p53 tumour suppressor gene in cell cycle arrest and radiosensitivity of Burkitt's lymphoma cell lines. *Cancer Res.* 53(20):4776-80.) For example, it has been shown that melanoma cells overexpressing a mutant form of p53 are more resistant to apoptosis thus possibly explaining why necrosis is a bigger factor in Sk-Mel3 cell death than in the AG1522 fibroblast cells that do have wild type p53. (Li, G., Bush, J.A., Ho, V.C. (2000) p53-dependent apoptosis in melanoma cells after treatment with camptothecin. *J Invest Dermatol.* 114(3):514-9.)

6.3.2 *Addendum to Chapter Four*

1. Q. Statement is confusing; are you saying that the sister clones have different responses to all treatments, with the exception of X-ray treatment, or are you saying that sister clones response to X-ray treatment has not been studied before? I believe you may need to reword the end of this sentence, as the next sentence seems to be answering my question. **See Chapter 4, page 152, paragraph 2.**

Answer.

The latter. Most studies look at chemical differences between cell clones derived from an untreated parental cell line but few studies examine x-radiation responses from sister clones derived from an untreated parental cell line. (See references 1-9)

2. **Q. There were 3 solvents described in this paragraph (in which the various drugs were dissolved). You need to mention whether the solvent itself independent of the drug, may affect the properties of the cells, which you are studying.** See Chapter 4, page 154, paragraph 3.

Answer.

I agree, the solvents can affect the properties of the cells (see comments on the solvent DMSO) but all drug experiments containing these solvents were compared with their corresponding controls without the drug but containing the solvent vehicle.

3. **Q. The normal human cell has 46 chromosomes. Does the parental HCT116 cell have this number of chromosomes? You are suggesting that this may not be the case, at least with the metaphase spreads described. If there was no (additional) genomic instability (with the cloned cells, compared to the parental cell line) – i.e. would you see 45 chromosomes, 46 chromosomes, or some other number?** See Chapter 4, page 170, paragraph 1.

Answer.

ATCC, the global nonprofit bioresource center that provided us with our cell lines (ATCC,_P.O.Box_1549_Manassas,_VA_20108_USA) describes the parental HCT 116 cells as epithelial-like with a near diploid stem line chromosome number (i.e. a modal number of 45). However, clones 2 and 10 do have a different compliment of chromosomes. As I have stated in the text "The chromosome numbers in 20 metaphases each of both clones 2 and 10 cells ranged from 39 to 46 with a peak of 44 chromosomes (Figures 3a I. and 3b)." See Chapter 4, page 161, paragraph 2. To answer the second part to your question, if there was no additional genomic instability or problems in chromosome separation during

mitosis, then you would see 45 chromosomes in the cloned cells compared to the parental cell line (which also contains 45 chromosomes). This is what you would expect since mitosis facilitates equal partitioning of replicated chromosomes into two identical groups.

4. **Q. Please cite the references for these statements. I am not sure if the references are the same, or not, as those mentioned within the first 4 lines of this page.** See Chapter 4, page 169, paragraph 1.

Answer.

The references have been cited. (i.e. **ref #10**. - Powell S, McMillan TJ. (1991) Clonal variation of DNA repair in a human glioma cell line. *Radiother Oncol.* Aug;21(4):225-32. **ref #11** - Leith JT, Dexter DL, DeWyngaert JK, Zeman EM, Chu MY, Calabresi P, Glicksman AS. (1982) Differential responses to x-irradiation of subpopulations of two heterogeneous human carcinomas in vitro. *Cancer Res.* 42(7):2556-61.)

5. **Q. As a continuation of point #3 above, the normal HCT 116 cells has 45 chromosomes. Does the published reports state the pathogenesis of the tumour cell line, led to the deletion of one chromosome?** See Chapter 4, page 170, paragraph 1.

Answer.

No, previous reports seem to suggest that it is the loss or gain of chromosome(s) or the parts of it that can lead to its pathogenesis. For example, it has been shown that genomic instability associated with deficiencies in mismatch repair (MMR) plays a critical role in tumourigenesis. (Aquilina G, Crescenzi M, Bignami M. (1999) Mismatch repair, G(2)/M cell cycle arrest and lethality after DNA damage. *Carcinogenesis*, 12, 2317-2326.) For example, HCT116 cell line is known to be deficient in DNA mismatch repair (MMR) due to a genetic defect in the hMLH1 gene, which is located on chromosome 3 and the loss of the ability of cells to repair mismatches in double-stranded DNA is a common finding in human malignancies (de las Alas, M.M., Aebi, S., Fink, D., Howell, S.B., Los, G. (1997) Loss of DNA mismatch repair: effects on the rate of mutation to drug resistance. *J. Natl Cancer Inst.* 89(20):1537-41) and that restoration of chromosome 3 has been shown to complement the *hMLH1* defect in the parental line and restores all mismatch repair activities. (Koi,M., Umar,A., Chauhan,D.P., Cherian,S.P., Carethers,J.M., Kunkel,T.A. and Boland,C.R. (1994) Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N-nitrosoguanidine tolerance in colon tumour cells with homozygous hMLH1. *Cancer Res.*, **54**, 4308–4312).

6.4 APPENDIX

6.4.1 *Calculation and normalization of survival curves*

Calculation of the unnormalized surviving fraction (SF):

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

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

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

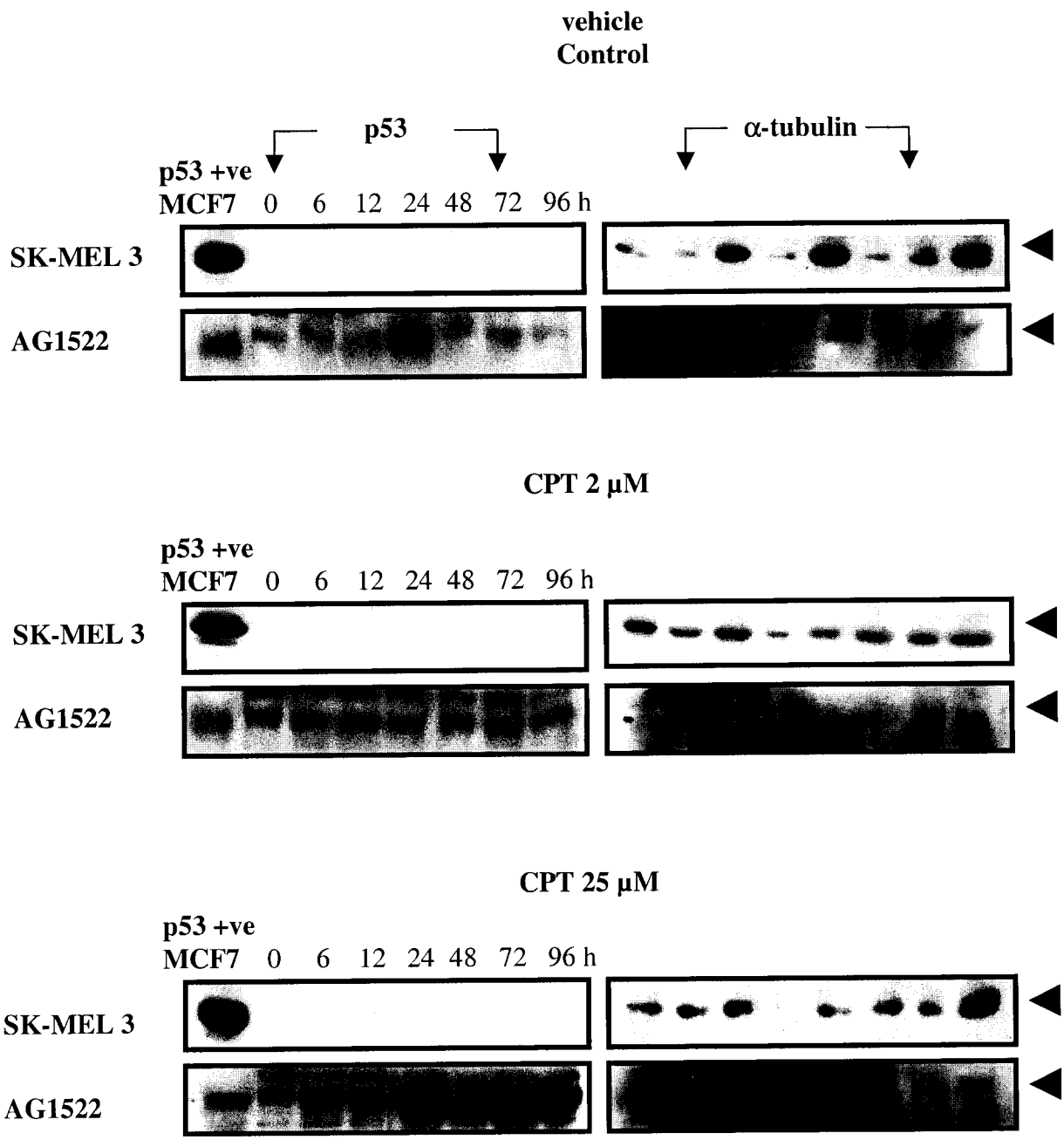
$SF_{normalized} = SF_{X\text{-}ray + CPT} / SF_{CPT}$

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

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

6.4.2 *Western blot analysis of p53 in Sk-Mel3 melanoma cells and AG1522 fibroblast cells treated with CPT.*

Figure 1. Western blots showing the expression of p53 after 1-hour exposure at 37°C to 2 μM and 25 μM of CPT in AG1522 fibroblast cells but not in Sk-Mel3 melanoma cells. Drug preparation, drug treatment, and immunoblot methodologies were performed according to the methods described throughout the dissertation.


vehicle
Control
p53
α-tubulin
p53 +ve
MCF7 0 6 12 24 48 72 96 h
SK-MEL 3
AG1522
CPT 2 μM
p53 +ve
MCF7 0 6 12 24 48 72 96 h
SK-MEL 3
AG1522
CPT 25 μM
p53 +ve
MCF7 0 6 12 24 48 72 96 h
SK-MEL 3
AG1522