IN VITRO CHARACTERIZATION OF SQUAMOUS CARCINOMA CELLS TO CISPLATIN AND RADIATION

THE IN VITRO CHARACTERIZATION OF A SQUAMOUS CARCINOMA CELL LINE TO COMBINED TREATMENT WITH CISPLATIN AND IONIZING RADIATION

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A Thesis Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree
Master of Science

McMaster University

MASTER OF SCIENCE (1996) McMaster University
(Physics) Hamilton, Ontario

TITLE: The *in vitro* Characterization of a Squamous Carcinoma Cell Line to Combined Treatment with Cisplatin and Ionizing Radiation

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NUMBER OF PAGES: xxviii, 200

Abstract

It is has been observed in several cell systems that cisplatin can radiosensitize and that the response of cells to combination cisplatin and radiation depends on several factors. These include the radiation dose and drug concentration used, the order in which the two treatments are administered, and the time between their administration. The response of a head and neck squamous carcinoma cell line to combination cisplatin-radiation treatment was examined. The response was found to be additive when cisplatin was given first, regardless of the timing and magnitude of the treatments administered. When cells were treated with radiation first, antagonism was observed for low radiation doses and drug concentrations. The response may be explained by a low radiation dose induction of processes that protect the cell from a second damaging agent, similar to the adaptive response. There is some indication in the literature that cisplatin can preferentially radiosensitize cells that are proficient in certain types of DNA repair. Therefore, the response of a cisplatin-resistant strain of the SCC-25 cell line was also investigated. The cisplatin-resistant cell line was found to be substantially radiosensitized by cisplatin for moderate amounts of radiation and cisplatin. The results are discussed with reference to the current proposed mechanisms for cisplatin-radiation interaction.

Acknowledgements

I would like to thank my supervisory committee for their time and effort on this project.

Thanks also to Susan, Ann and Roger for sharing their laboratory expertise, their scientific wisdom, and their friendship. Their strength of character and devotion to science will inspire me always.

Thanks to Myrna who always put the troubles of others before her own and still managed to keep an enthusiastic smile.

Thanks to Rob, Rob and Tanya for encouraging words, invaluable brainstorming sessions, and lots of coffee.

Thanks to David, Fiona and Joanne for listening, and for unwavering support and encouragement that has kept me in the field of science.

Thanks, last but certainly not least, to my family, whose unconditional love and support gives me the strength to be the person I am.

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List of Abbreviations

SCC	Squamous cell carcinoma
CDDP	cis-diamminedichloroplatinum(II)
Cisplatin	cis-diamminedichloroplatinum(II)
DRI	dose reduction index
CI	combination index
DNA	deoxynucleic acid
Ssb	single strand breaks in DNA
uM	micromolar
Gy	gray
IRR	induced radioresistance
HRS	hyper-radiosensitive

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Introduction

The Antitumor Action of Cisplatin

Cisdiamminedichloroplatinum (cisplatin) was discovered during a test of the effect of an electric field on growing E. coli. An inhibition of division but not of cell growth was observed. The effect was not due to the electric field but to cisplatin which was produced when the platinum electrodes came in contact with ammonium chloride in the cell culture medium [1,2]. In 1968 an intuitive step was made to test cisplatin for anti tumor activity in an animal model where the drug was seen to inhibit a solid sarcoma-180 tumor [3]. The first report of clear anti tumor effect came by Hill et al [4]. Since the discovery of its anti tumor activity, cisplatin has proven to be effective against a broad range of animal tumors [5] and against a variety of human malignancies, including head and neck, testicular, bladder and ovarian cancers [5, 6, 8].

Cisplatin is a square planar molecule consisting of two ammine and two chloride groups arranged in the cis configuration in figure (i)[68]. Cisplatin remains in a relatively inactive molecular form until it passively diffuses[9,10] into the cell, although active transport may also be involved[11,12]. Since most intracellular chloride concentrations are

low relative to the extra cellular fluid the two chloride groups of cisplatin easily leave the parent compound once inside the cell. Immediately following the leaving of the chloride groups, the parent cisplatin compound is rapidly aquated with the addition of water and hydroxyl groups[13,14]. It has been determined that the theoretical aquation rate is equivalent to the rate of protein-cisplatin binding[15] and the activation enthalpy for cisplatin aquation is the same as that for cisplatin-DNA interaction and viral inactivation of DNA by cisplatin[16,17]. Therefore, the aquation reaction is deemed the rate limiting step for biological interaction and can be thought of as an intracellular activation event for the drug.

Once aquation has occurred (figure (ii))[68], the electrophilic cisplatin equilibrium products are attracted to electron rich sites of various macromolecules. Cisplatin concentrations are highest in the cytosol, microsomes and the nucleus[18]. Cytosolic platinum is mostly bound to metallothioneins (MTs) and glutathiones (GSHs) which are heavy metal scavengers that protect the cell against damage like DNA strand breaks which occur as a result of normal metabolic function and assault by external agents[19]. The MT and GSH groups are thought to protect against cisplatin damage however this protection is limited since MT and GSH production can not be induced by cisplatin[15]. Platinum is known to bind to RNA, DNA and protein[10,20]. The critical target for cellular damage by cisplatin is believed to be nuclear DNA[10, 21, 22]. This conclusion is supported by observations that the inhibition of DNA synthesis by cisplatin is achieved for drug concentrations lower than those required to inhibit RNA and protein synthesis [22]. Further

evidence is provided by the observed correlation between cytotoxicity and the amount of DNA-platinum binding[22]. It has also been observed that cells deficient in DNA repair are more sensitive to cisplatin than wild-type cells[100, 20].

Cisplatin is believed to interact with DNA in a two-step process. One arm of aquated cisplatin binds covalently to nucleophilic sites of DNA to form a monofunctional adduct followed by the binding of the second arm to produce a bifunctional adduct[22, 26]. The bifunctional adduct can be between base pairs within the same DNA strand, an intrastrand crosslink, with opposite DNA strands, an interstrand crosslink, or with one DNA strand and a protein[27,28]. The most abundant adducts are intrastrand which comprise approximately 90% of all crosslinks[29,30]. These adducts are thought to be formed between the most nucleophilic sites of guanine and adenine, the N7 sites[31]. Interstrand crosslinks and protein crosslinks make up between 1% and 5% of total crosslinks[32]. The interstrand crosslink is believed to be between the two N7 sites on guanine or between guanine and cytosine. Since cisplatin will bind with any nucleophilic sites of DNA many reaction products can result. Only some of the products are stable and others last approximately 30 days. The relative toxicity of the different lesions has yet to be established[32]. In the first few hours after cisplatin treatment the number of bifunctional crosslinks continues to increase as the second arm of unrepaired monofunctional adducts slowly crosslink with DNA. Repair of adducts is ongoing during and after treatment.

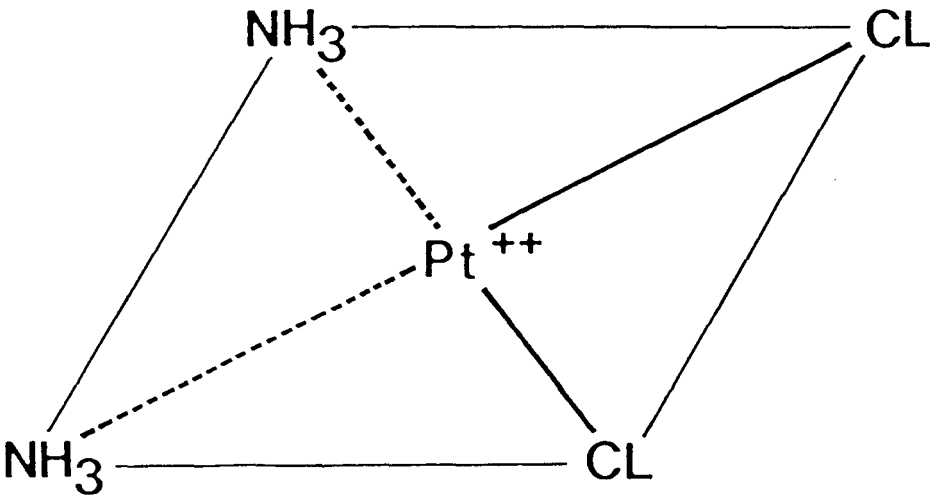


Figure (i): the structure of cis-diamminedichloroplatinum (II).

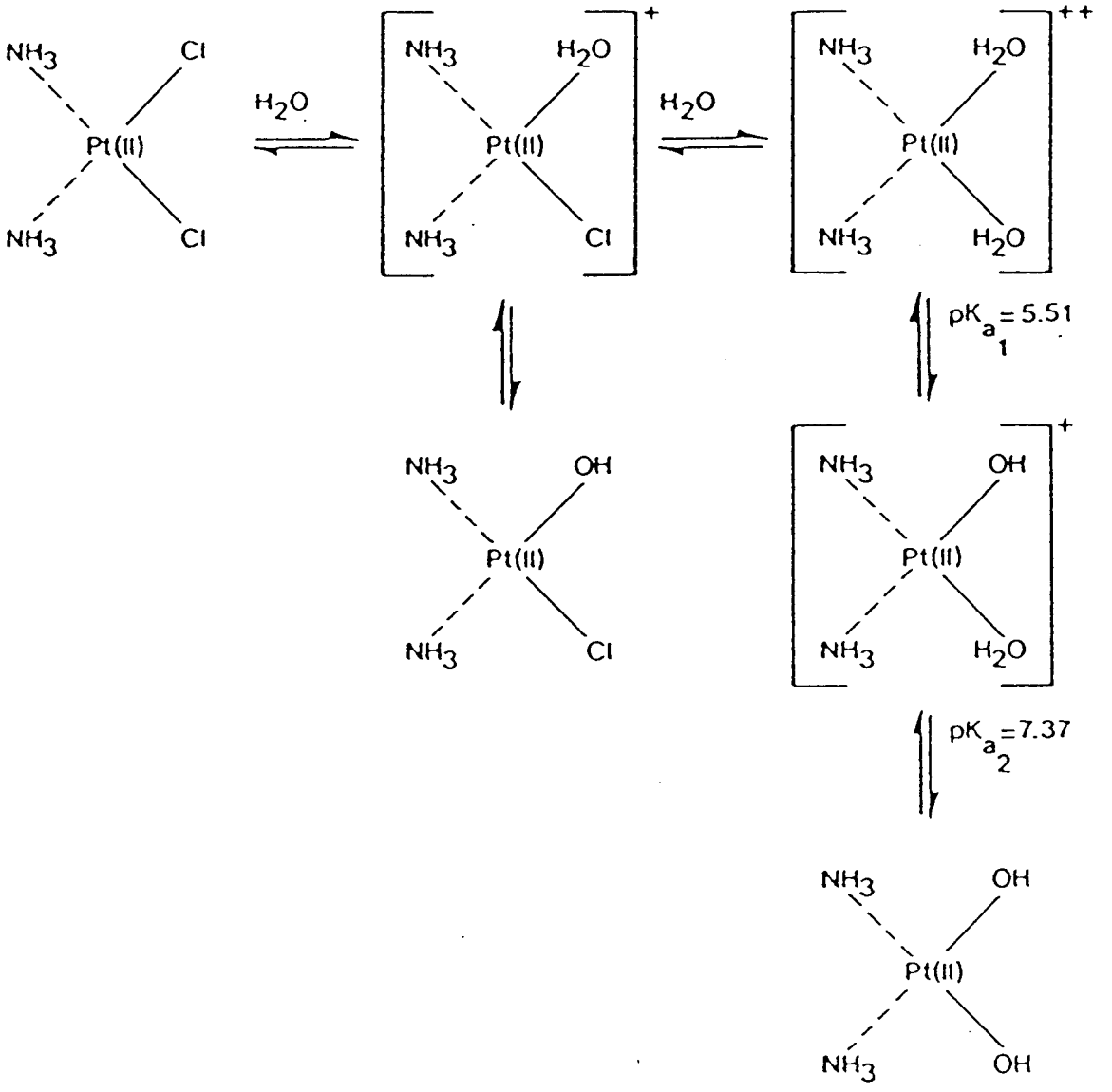


Figure (ii): Aquation of cisplatin.

Cisplatin-DNA crosslinks are believed to interfere with DNA replication[33, 34, 15]. Inhibition of transcription and translation by cisplatin does not occur[38, 39]. It has been determined that the formation of DNA precursor molecules and their transport through cellular membranes are not affected by cisplatin[38]. Furthermore, ATPases[15, 42] and DNA polymerases required for DNA synthesis are not inhibited by the drug[38, 40, 41]. Therefore, inhibition of DNA synthesis is thought to result from direct interaction of cisplatin and DNA.

The exact mechanisms of cisplatin-DNA adduct removal are not known. However, both long and short patch excision repair have been implicated in the repair of cisplatin-DNA damage[20, 43, 45, 48]. Excision repair involves recognition of damage, incision near the damage, removal of the damaged stretch, and polymerase filling of the gap with ligation of the two DNA strand ends[19].

If the cisplatin-DNA adduct burden is great enough, the repair of bulky cisplatin-DNA adducts or single and double strand DNA breaks produced during repair can remain incomplete[19]. These lesions interfere with replication and consequent DNA synthesis causing mutations that can lead to cell death or termination of the cell's ability to proliferate. Cisplatin is able to cause more damage to tumor cells than to healthy cells because tumor cells progress through the cell cycle at a more rapid rate than healthy cells and the checkpoints for repairing cellular damage are often bypassed. Besides targeting tumor cells via their rate of cell-cycling, cisplatin accumulates in the kidneys, liver, lung and ovaries allowing targeting of cancers in these organs[46].

The Antitumor Action of Radiation

Radiotherapy has been successfully used in the treatment of cancer for the past 30 years. The critical target of radiation for cellular damage is believed to be DNA. Evidence for this includes experiments in which microbeams or cellular incorporation of specific radioisotopes have been used to irradiate the cytoplasm or nuclei of cells. This idea is supported by the finding that cells deficient in DNA repair are radiosensitive[47, 48]. Excision repair is thought to be especially important in the repair of radiation-induced damage[49].

Radiation is known to cause single and double strand DNA breaks by directly interacting with the DNA sugar-phosphate backbone structure. A double strand DNA break can be produced by a single event or by two single strand breaks close together[19]. Radiation of low LET (linear energy transfer), such as x-rays or γ-rays, more frequently produces biological damage by interacting with water molecules to create free radicals. These free radicals can attack the sugar-phosphate backbone of DNA and directly produce single and double strand breaks[50]. Free radicals can also react with the bases of DNA altering their electronic structure[19] which may result in the loss of a base or in the formation of DNA-DNA cross-links or DNA-protein cross-links[51]. As occurs with cisplatin, the conformational changes in the DNA structure caused by radiation are repaired by a sequence that begins with the recognition of lesions and proceeds with the cleavage of DNA by enzymes to form strand breaks. So DNA strand breaks can be

formed indirectly. In general, double DNA strand breaks are thought to be the most lethal of the lesions produced by radiation[52]. Single DNA strand breaks are not usually lethal since the second DNA strand can act as a template for the repair of the missing or damaged information. Conversely, when a double DNA strand break occurs, which is a less frequent event than a single strand break occurrence, there is nothing to keep the two sets of genetic information from being spatially divided and there is, consequently, no template for repair[19].

The result of DNA cross-links and strand breaks is often genetic mutations that are lethal to the cell. When high doses of radiation (10-100 Gy) are given, radiation-induced damage can inhibit DNA, RNA, and protein synthesis, leading to cell death. Cell death can also occur if radiation results in a lethal chromosomal aberration, which can occur for radiation doses of less than 1 Gy. Chromosomes can be broken by the direct or indirect interaction of radiation. Aberrations occur when re-attachment of the chromosomal segment is incorrect[19].

Radiation causes more damage to tumor cells than to healthy cells because tumor cells progress through the cell cycle at a more rapid rate than healthy cells and the checkpoints for repairing cellular damage are often bypassed. Besides targeting tumor cells via their increased metabolic rate, radiation can often be delivered locally so that tumor cells are targeted by close spatial proximity of the radiation source to the tumor site. This also allows the majority of healthy tissue to be spared.

Combination Cisplatin and Radiation Treatment

Cisplatin is currently the most effective chemotherapeutic agent in the treatment of cervical squamous cell carcinomas with a response rate of 50% in tumors that are detected in the early stage of disease[53] . The survival at five years for patients with advanced tumors is less than 35%, depending on the stage of disease. The response rate for cisplatin in the treatment of recurrent squamous cell carcinoma (SCC) of the head and neck is less than 45%, depending on the stage of disease[53]. Similarly, squamous cell carcinomas of the upper respiratory and digestive tracts have been effectively treated with radiotherapy when diagnosed in the early stage of disease[54]. Prognosis for patients with advanced disease is poor.

Current approaches to the treatment of cancer include spatial separation of tumor and normal tissue through the uses of radiotherapy, surgery and hyperthermia and a dependence upon the innate differences in response between tumor and healthy cells to systemic treatments like chemotherapy[55]. In the cases where disease is advanced, one alternative is to combine chemotherapy and radiotherapy. Combination treatment can provide a clinical advantage if the two treatments improve cell kill and have toxicities that are different. This can significantly decrease patient toxicity, which is the limiting factor for a given cancer treatment. The combination of cisplatin and ionizing radiation is promising because cisplatin toxicity is mainly renal[56-58], gastrointestinal, and in myelosuppression [15]. The toxicites of radiation are more local than systemic for typical treatments and depend on the part of the anatomy targeted, and on the method of

radiation delivery (brachetherapy or teletherapy). Therefore, the overlap in toxicities from cisplatin and ionizing radiation is relatively small[59, 60].

However, combination treatment with two agents may not necessarily result in increased cell kill relative to either agent alone. Two agents in combination can interact by one or more of the following mechanisms. One agent can react with an intermediate species which alters the ability of the second agent to reach its target for biological effect. One agent can react with an intermediate species which alters the activity of the second agent after it reaches its target. One agent can affect cell cycle kinetics which alters the biological effect of the second agent after it reaches its target. Both agents can reach different targets, resulting in effects that complement, potentiate or antagonize one another. Both agents can combine with one another to produce a new agent which has biological effects, and finally, both agents can reach the same target[61]. The result of combination treatment for any two agents is difficult to predict because molecular mechanisms of interaction are multifactorial and, therefore, complex. The determining factor in whether a combination is applied clinically or not, is the outcome of *in vitro* studies, *in vivo* animal studies, and a series of clinical trials, for the combination.

Radiosensitization was first observed for the combination of cisplatin and radiation when cisplatin and whole body irradiation produced an increase in mouse survival[62]. This initiated an investigation into the combined effects of cisplatin and radiation in a variety of experimental models. Numerous studies have been done on different types of cultured mammalian cell lines and in different model systems. The

results are largely inconsistent between investigators even for a given cell line and similar experimental conditions[63-65]. Some investigators observed radiosensitization that depended on the cells being hypoxic[64-67]. Others observed radiosensitization only in aerobic cells[68] and still others observed no dependence of radiosensitization on oxygen status[69, 70]. The importance of the phase of growth of treated cells (plateau or exponential) is also in contention[66]. In general, it has been shown that cisplatin can produce radiosensitization in a variety of mammalian cell lines[70, 71] and in some animal tumor models[68]. It is generally agreed that the degree of radiosensitization depends on the oxygenation status of the cells, the magnitude of the radiation dose and drug concentration given[72, 69], the sequence in which the two are administered[70, 73, 74], and the time between their administration[74, 19].

Since the degree of radiosensitization depends on the sequence of administration of cisplatin and radiation, it is feasible that the damage caused by the first agent is influential in determining the damage caused by the second agent. The influence of the first agent must be relatively transient since the time between the administration of the two agents plays a role in the response. These observations have lead to the investigation of different mechanisms of combination cisplatin-radiation interaction. The mechanisms of interaction that are currently believed to play a role in combination treatment are cell cycle kinetics and the repair of DNA damage.

Every population of cells that is undergoing exponential growth is composed of individual cells randomly distributed in different phases of the cell cycle (mitosis, G_1,

DNA synthesis, G_2). There is some evidence that the first agent to be administered in radiation-cisplatin combination treatment, to a randomly distributed population could cause a re-distribution of the cells into a phase that is sensitive to the second agent[72, 75]. This occurs because there are certain phases of the cell cycle for which cells are more sensitive to cisplatin or radiation. For example, if a population of cells is relatively resistant to radiation in late S phase (synthesis) and sensitive to radiation in G_2 and M (mitosis) phases, which is typical of mammalian cell lines, then cells in the resistant phase will have a higher probability of survival following radiation treatment then those in the sensitive phase. Therefore, following radiation the cells will be partially synchronized in the cell cycle. If the cell population being studied can be synchronized by radiation into a phase of the cell cycle which is cisplatin sensitive, then the addition of cisplatin as the second agent will optimize cell kill[76]. Similarly for radiation as the second agent. Since the rate at which each cell progresses through the cell cycle varies, the synchronization decays quickly. This could explain why the time between administration of the two agents is important in cell response.

Another mechanism proposed for the combined action of cisplatin and radiation is the inhibition of radiation-induced damage repair by cisplatin. It has been shown that cisplatin can inhibit the repair of radiation-induced damage[66, 68, 77, 78, 73, 95] even when administered after radiation treatment[73, 71]. The degree of repair inhibition is dependent upon the sequence and timing of the combination treatment[73]. Although the mechanism by which cisplatin inhibits cellular repair is not known[68, 73], the repair inhibition model is

relatively consistent with the data that already exists for combination cisplatin-radiation treatment.

It has recently been determined that an elevated repair capacity plays a role in radiation resistance, so the use of cisplatin to inhibit proficient radiation repair may provide an effective option in the treatment of radiation resistant tumors[71, 79]. A major limitation of clinical efficacy of cisplatin is intrinsic acquired resistance[80]. For some tumor types, especially in head and neck tumors[81], cisplatin resistance is correlated with radiation resistance. This implies repair proficiency in both radiation and cisplatin resistance. It would, therefore, be advantageous to know if cisplatin retains its ability to inhibit the repair of radiation damage in cells that are cisplatin resistant[82]. If so, combination cisplatin-radiation treatment could be effective in the treatment of cisplatin-resistant tumors.

The efficacy of combined cisplatin-radiation treatment has been clinically investigated in several studies. Some studies have found cisplatin to be a radiosensitizer[68, 83, 84]. Others have found no sensitization by cisplatin to radiation[85-87], or a possible increase in patient survival with a marked increase in toxicity[84]. The results of well controlled phase III clinical trials involving combination treatment are not yet known. Most of the clinical studies have looked at combination cisplatin-radiation as a treatment for advanced stage disease, and not as a treatment for tumors resistant to radiation or cisplatin. Therefore, experimental evidence supporting or contraindicating

combination cisplatin-radiation as a treatment for resistant disease could be very helpful in determining the future clinical direction of combination cisplatin-radiation treatment.

In light of a new and recent interpretation of low dose radiation survival data and combination treatment involving low radiation doses, there exists another possible outcome to combination treatment that is similar to the adaptive response by radiation. The outcome is an increase in tumor cell survival when combined treatment is administered.

The Adaptive Response

It has been observed that pretreatment with relatively low doses of radiation can increase the resistance of cells to subsequent radiation exposure. This effect has been observed in plant[127], single cell[120, 128], hamster[129], and human lymphocyte[121] systems, and is commonly referred to as the adaptive response. It has been determined that a variety of DNA lesions can produce the adaptive response and that the effect can be inhibited by cyclohexamide, an inhibitor of protein synthesis[121, 130-132], suggesting that the effect involves the induction of repair processes. The exact mechanism of the adaptive response is not yet clear, however, the existence of an inducible response to radiation is now generally accepted.

Over the past forty years investigators have identified two distinct components in the low dose region of radiation survival curves in a variety of cell systems, including hypoxic hamster[134, 142], mammalian[133, 143], normal human[117] and human tumor[118, 145] cell systems. The cells in these models are typically hypersensitive to radiation doses less than 1 Gy. For radiation doses above 1 Gy, cells are more resistant per unit dose. Some investigators have more specifically defined the region over which hypersensitivity occurs. For hamster cells, the hypersensitivity region has been defined as being between zero and 0.3 Gy[133]. In V79 hamster cells, the sensitive region is between zero and 0.2 Gy[133]. In general, the survival curve is characterized by a relatively steep decline in survival in the region from zero to about 0.5 Gy , followed by a more gradual decrease in survival for doses greater than 1 or 2 Gy. The region in between the hypersensitivity and increased resistance is either a plateau[146] or a region of upwards concavity[118], depending on the cell line and assay used. The region of hypersensitivity has commonly been referred to as the hyper-radiosensitive or HRS region. The section of the survival curve just following the HRS region, in which survival increases, has been named the region of induced radioresistance or the IRR region[133, 134].

Certain investigators have suggested that the biphasic survival response is due to the presence of one or more radiation resistant sub-populations in the sample[118, 133, 143]. By this theory, the HRS region would reflect the death of cells in sensitive phases of the cell cycle, and the IRR region would reflect cells in relatively resistant phases of the cell cycle, since these cells would require a greater dose per unit kill than the sensitive cells. There is evidence that the HRS and IRR regions cannot be explained by sub-populations

of different radiosensitivities. This evidence, as discussed by Skov and Marples[139], includes the finding that some biphasic survival curves have an upwards concave region between the HRS and IRR regions[118, 139]. An increase in survival, such as this, is not consistent with the theory of heterogeneous populations. In addition, survival curves with both the HRS and IRR regions have been produced for synchronized cell populations in V79 hamster[133] and human cells[135]. Finally, the heterogeneous population theory is not consistent with the observation that the extent of kidney damage in mice given 0.75 Gy fractions of x-radiation is greater than the damage produced for higher dose fractions[119, 120].

An alternative hypothesis is that at some critical radiation dose, a biological mechanism for dealing with radiation is triggered. Any radiation dose that does not meet the critical dose is particularly lethal per unit dose and is characterized by the HRS region of the survival curve. Once the radiation dose reaches the threshold for inducing the protective response, radiation is not as effective in producing cell death and the survival curve shows a region of IRR.

It is known that DNA damage can trigger the inducible response. This is not surprising considering the well established finding that DNA damage plays a significant role in cell death due to radiation. Evidence for repair as part of the inducible response comes from data with V79 hamster cells. The addition of agents known to inhibit repair,[136, 137, 121, 125], through various pathways, following radiation, decreases the IRR response. At least two of these agents, cyclohexamide and 3-aminobenzamide, can also

eliminate the adaptive response in V79 hamster cell lines[121, 125]. In addition, host cells pretreated with gamma and ultraviolet radiation have an increased ability to reactivate virus damaged by radiation[125, 147, 109], implying that repair of DNA damage is increased in the adaptive response. More specifically, excision repair has been suggested as a trigger[138] for an induced response. However, this may merely reflect the role of ssbs that are produced during excision repair[139]. It is thought that excision repair or the ssbs, can trigger any one or a number of induced systems causing the increased resistance. These systems include an increase in the production of repair enzymes or the production of improved enzymes and, therefore, increased repair[122]. Alternatively, a cell cycle delay may be induced, allowing more time for the repair of DNA damage, and increased survival[123]. Not to be discounted are up-regulation of growth factors, the inhibition of apoptosis[139] and the role of gene expression in these processes[140, 122].

Similarities Between The Adaptive Response and The IRR Response

One might expect that if an agent that inhibits repair can suppress IRR, then an agent that triggers repair might increase the IRR response. This has been observed in hamster cells. Pretreatment of hamster cells with 0.2 Gy of x-rays before a challenge dose is known to induce IRR and abolish the HRS region[125]. This effect is dose dependent and is less apparent for pretreatment doses greater than 0.2 Gy. Colony size has been shown to be biphasic for single x-ray doses[124] and when a priming radiation dose is followed by a challenge dose, colony size is seen to increase in V79 hamster cells. Other DNA damaging agents reported as having induced a radioprotective effect against

subsequent radiation exposure include tritiated and C-14 labeled thymidine[139]. Non-toxic doses of hydrogen peroxide can induce both IRR[125] and an adaptive response in human cells[126, 121]. Cisplatin can also induce both the IRR[72] and adaptive responses[135, 148, pers. comm. Skov,1996].

The dose dependence for radiation pretreatment of hamster cells before subsequent radiation exposure is further evidence of a threshold for the induction of a protective system[125, 141, 148]. Experiments with hydrogen peroxide also support this theory. Concentrations of hydrogen peroxide below 10^{-4} M do not result in radioprotective action for the V79 cells[125]. The time dependence of IRR is consistent with the time dependence of the adaptive response. Pretreatment with x-rays 6 hours before a challenge dose induces IRR, eliminating the HRS region. When the challenge dose is given concurrently or at 24 hours (2-3 cell cycles in this case), IRR is not induced[125, 141]. The adaptive response also typically lasts 2-3 cell cycles. The adaptive response is known to peak in the 5-6 hour range[127] for alga and in 6-8 hours for V79 hamster cells[125]. The response fades by 24 hours. The IRR response in mammalian cells has been reported as disappearing 7-8 hours after induction[149].

The time course for both the adaptive response and the IRR response is comparable. Both responses are inhibited by the same agents, implying a common pathway of induction, and both responses can be triggered by adding DNA damaging agents above some threshold dose. Several investigators have noted the similarities between the phenomena of IRR and the adaptive response. It has been proposed that the

IRR and the adaptive response may be manifestations of a common inducible protective mechanism. Determination of the mechanisms underlying both the IRR and adaptive responses would help to establish whether or not the two responses are indeed manifestations of the same phenomenon.

Clinical Applications

The ability of various agents to induce or inhibit IRR illustrates the need for further investigation of this response since combined modality therapy could be gravely affected. Subjects important in the development of an optimal clinical treatment include the timing between the two agents in combined modality and the relative amount of each agent. Agents known to induce IRR could be avoided in combined modality treatment. In addition, several researchers have noted a correlation between the intrinsic radiosensitivity of various human tumor cell lines and the extent of IRR observed. For example, decreased IRR is observed in human tumor cell lines that are clinically radiosensitive and in hamster cells that are DNA repair deficient[151]. This might suggest that radioresistance is an induced effect. If this is true, then clearly radioresistance of tumors may be avoided if appropriately small amounts of DNA damaging agents are used when the tumor is initially treated. Radioresistance might also be treated by an agent that can inhibit IRR. Other clinical applications, such as improved radiation treatment by administration of very small radiation doses targeting the hypersensitive region of cell response, and an improved understanding of normal tissue damage and the induction of

secondary cancers in the tissue surrounding the tumor being treated are discussed by Marples and Skov[139] .

Hypothesis and Objectives:

Hypothesis:

Combined treatment of head and neck squamous carcinoma cells with cisplatin and ionizing radiation results in more cell death than treatment with either agent alone.

Objectives:

1. Compare combination treatment to radiation treatment.
2. Compare combination treatment to drug treatment.
3. Determine if cisplatin and ionizing radiation interact synergystically.

It is well documented in the literature that cisplatin can be a radiosensitizer and that the response of cells to combination cisplatin and radiation depends on several factors. These include the amount of each treatment, the order in which the two treatments are administered, and the time between their administration. Therefore, the objectives were met for four treatment schedules which investigate the effects of treatment dose and concentration, timing, and order on the response of the head and neck SCC-25 cells. The four schedules are listed in table 1.

A proposed mechanism for cisplatin radiosensitization is the ability of cisplatin to inhibit the repair of radiation-induced damage. There is some indication that cisplatin may be able to preferentially radiosensitize cells that are proficient in certain types of DNA repair. A fourth objective was defined for the last of these treatment schedules in order to determine whether combined cisplatin and radiation might be effective in decreasing survival in cisplatin-resistant cells.

Objectives continued:

4. Determine if cisplatin-resistant cells derived from the head and neck squamous carcinoma cell line have a synergystic response to combination treatment.

Schedule #	Treatment #1	Wait	Treatment #2
1	Cisplatin	10 minutes	Radiation
2	Cisplatin	60 minutes	Radiation
3	Radiation	10 minutes	Cisplatin
4	Radiation	60 minutes	Cisplatin

Table 1: The order and timing schedules for cisplatin and radiation administration investigated in this study.

Materials and Methods

Cell Lines

The SCC-25 and cisplatin-resistant SCC-25/CP cells are human squamous carcinoma cells (SCC) of the tongue. The SCC-25 cell line was obtained from Dr. John Lazo, Department of Pharmacology, University of Pittsburgh School of Medicine. The SCC-25/CP cell line was developed by Teicher et al of the Dana-Farber Cancer Institute, Division of Cancer Pharmacology, Boston, MA, USA by repeated exposure of SCC-25 cells to increasing concentrations of cisplatin[88]. Both cell lines were grown as monolayers in Dulbecco's Modified Eagle Medium (DMEM-high glucose), supplemented with 10% Fetal Bovine Serum, 0.4 ug/mL final concentration hydrocortisone (dissolved in 10% ethanol) and 1% antibiotic-antimycotic solution (penicillin G sodium 10 000 units/mL, streptomycin sulfate 10 000 ug/mL, amphotericin B as Fungizone [registered trademark] 25 ug/mL in 0.85% saline) all from GibcoBrl Laboratories, Burlington, Ontario, Canada.

The SCC-25 cell line was chosen because of its comparability in type (SCC) and origin (head & neck) to tumors treated with combination radiation-cisplatin in several clinical studies[83, 70, 91]. No experimental *in vitro* work with combination cisplatin and radiation has been done on this cell line to date.

Colony Forming Assay

Cells were grown to semi-confluency in supplemented DMEM, suspended by application of 2 mL of 10X trypsin-EDTA (trypsin 0.5%, EDTA.4Na 5.3 mM from GibcoBrl) and pelleted by centrifugation for 5 minutes at 1000 RPM and 20 degrees Celsius (IEC Centra-8R centrifuge, USA). Cell pellets were re suspended in supplemented media, counted using a hemacytometer and serially diluted for seeding. Cells were seeded at a density of 1800 SCC-25 cells per well and 1000 SCC-25/CP cells per well in Corning 6 well plates (Fischer Scientific, Mississauga, Ontario, Canada).

Cells were incubated overnight before treatment to ensure their adherence to the plate. After treatment cells were incubated at 37 degrees C and 5% carbon dioxide in 95% humidity. Cells were permitted to grow for 5-7 days depending on colony growth in control samples. The doubling time for the SCC-25 and SCC-25/CP cells was approximately 28 hours each. Experiments in which control samples contained colonies of at least 20 cells defined as having reached the end of the assay. Media was then

removed from each well, samples were rinsed gently with PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2, from Fischer Scientific), and flooded with 0.5% methylene blue stain (Sigma Chemical Co., St. Louis, MO, USA) dissolved in 70% methanol, 30% distilled water. Using a light microscope, a colony of 10 or more cells was counted as a survivor.

Tumor growth can be halted if tumor cells lose their ability to divide. The colony forming assay is based on this concept and measures the loss of tumor cell reproductive ability. The term survival in the context of this assay refers to cells that have retained the capacity for sustained proliferation following treatment. That is, after treatment the samples are maintained in a fixed environment for a specific length of time. Each cell that has retained the ability to proliferate proceeds through the cell cycle and divides. The daughter cells attach to the bottom of the petri dish next to the parent cell and after many divisions, a group or colony of cells is formed for each original survivor of the treatment. The number of survivors is counted for a given treatment sample and compared to a control sample to determine the relative surviving fraction. Both the SCC-25 and SCC-25/CP cell lines grow as colonies in monolayer (attached to the bottom of the petri dish) which qualifies them for use in this assay.

The number of colonies counted in each sample relative to the control, depends on the treatment given, the conditions the sample endures post-treatment, and the length of time for which the sample is allowed to grow. Within a given experiment, all samples

see the same post-treatment conditions, such as incubator humidity, temperature and carbon dioxide level. Samples in the same experiment will also grow for the same length of time. The treatment seen by each sample is well quantified, as discussed above for each of cisplatin and radiation treated samples.

However, samples in different experiments may experience different conditions in the incubator. Samples seem to grow more slowly when there are many samples present in the incubator at once. This could be due to ineffective circulation of carbon dioxide. The conditions of constant temperature and humidity in the incubator can be upset when the door is opened, so if several experimenters are using the incubator during the growth time of the colony forming assay, colony growth can be affected. Therefore, it is not valid to set the time of the colony forming assay in number of days. The samples were consequently allowed to grow until the majority of the control colonies consisted of 20 cells. This corresponded to just over 4 cell doublings and between 5 and 7 days, since the doubling time for both the SCC-25 and SCC-25/CP cell lines is approximately 28 hours at the seeding density used. This allowed survival to be standardized between experiments. The number of cells per colony at the end of the assay was consistently smaller in the samples that received high radiation doses and large drug concentrations. This is probably because cell cycling is stalled following treatment so that cell repair can take place. The criteria for a colony to be counted as a survivor of the treatment was 10 cells per colony.

The number of cells seeded for the assay depended on the plating efficiency of the cell line which is usually quoted as the percentage of cells that adhere to the plate compared to the number of cells seeded. The plating efficiency is approximately 20 % for the SCC-25 cell line and 35% for the SCC-25/CP cell line. The plating efficiency for both cell lines decreased dramatically when less than 500 cells were seeded. The plating efficiency gradually increased between 500 and 8000 cells. The upper limit on the number of cells seeded was set to avoid crowding in the dish and consequent overlapping of cell colonies which made it difficult to accurately count the number of surviving colonies.

The points plotted on the graphs and analyzed were taken as the mean of three samples of size n=1. The error bars are the standard error of the mean. The error bars, therefore, represent the fluctuation in cell survival due to differences in the 'fixed' conditions (incubator) between each experiment, differences between the batches of media used for each experiment, and in the pipetting of a fixed number of cells during seeding of each experiment. Any instability in the cell characteristics for the duration of the project is also accounted for by the error bars, although the radiation and drug only survival curves showed no substantial variations over the duration of the project.

Cisplatin Treatment

Cisplatin was purchased from Sigma Chemical Co., St. Louis, MO, USA and was prepared as a 333 uM stock solution in sterile PBS prior to each experiment. The solution was sterilized by filtration through a 0.2 micron Acrodisc (Gelman Sciences, Montreal, Quebec, Canada). Appropriate concentrations of drug were serially diluted from the stock solution using fresh supplemented media. Each sample was incubated with the appropriate cisplatin concentration for a duration of 1 hour while controls saw fresh media for the same time. At the end of cisplatin treatment the drugged media was removed and each sample received fresh supplemented media.

Administration of the drug to each sample was done by serial dilution from a stock solution so that each sample receives an appropriate amount of drug relative to the highest drug concentration. For example, the 10 uM sample receives half the drug concentration of the 20 uM sample. The method of serial dilution is accurate and precise, so the relative drug concentrations within an experiment can be judged with confidence.

The difference in absolute drug concentrations between experiments was too small to detect with the colony forming assay. This conclusion was reached by comparing the drug survival curves for each experiment which were comparable within error.

Radiation Treatment

Irradiations were conducted with a ^{60}Co source (Theratron-80). The dose rate for the isotope was 0.681 +/- 0.003 Gy/minute in June of 1995 when experiments were first started and decreased by a total of 0.083 Gy/minute by June of 1996 upon completion of experiments. All samples were placed inside plastic boxes and sealed before leaving the laboratory for irradiation. This was done in order to reduce the opportunity for sample contamination. The lid thickness of the boxes was 1.90 +\- 0.01 mm, and the 6-well plate had a lid of thickness 1.82 +\-0.01 mm. No correction was made for the attenuation from the lids because the difference in cell survival with and without the lid present was not measurable by the colony forming assay. The plastic boxes were positioned at 80 cm from the source centered in a field size of 30 cm by 30 cm. The cells grew in monolayer in a volume of 5 mL. This corresponds to a depth of approximately 1 cm from the surface of the medium. Therefore, the cells are located at a distance into the media where they receive 98.5% of the maximum dose (D_{max}). The dose rates quoted above are already corrected for this deviation from D_{max}. The time for which each sample was exposed to the source defined the dose to each sample and was controlled from an operating table outside the irradiation room.

All samples from a given experiment, including controls, were taken to the irradiation room together and therefore were removed from controlled temperature, carbon dioxide level and humidity for the same length of time. All samples except those

undergoing irradiation were kept outside the treatment room during the length of the irradiation procedures.

The relative radiation doses within an experiment, which were determined by taking the known dose rate of the ^{60}Co source, correcting for source decay using an exponential decay equation, and multiplying by the exposure time, were also well known. The half-life of the ^{60}Co source is sufficiently long that the dose rate can be treated as a constant over the duration of the treatment. Therefore, a sample that is exposed to the source for twice as long will receive twice the radiation dose.

The difference in absolute radiation doses between experiments was too small to detect with the colony forming assay. This result was determined by comparing the radiation survival curves for each experiment which were comparable within error.

Combined Treatment

Radiation and drug treatment were carried out according to four different schedules. The schedules are organized according to the treatment given first (treatment #1), the treatment given second (treatment #2) and the time interval in between the two treatments (wait). The schedules are listed in table 2 according to experiment number:

The samples in each schedule were divided into two groups for irradiation. The samples receiving radiation doses of 0.05 to 1.0 Gy were in the low dose group, and samples receiving 2.5 and 7.0 Gy were in the high dose group. Irradiations were timed so that the higher doses in each group, and therefore the longer exposure times, went first. This ensured that the 10 minute time interval between the end of radiation and the beginning of drugging could be met for all samples.

The outcome of combination radiation-cisplatin treatment in vivo and in vitro has been known to change according to several factors. These factors include: the order of administration, the time between administration, the dose or concentration administered, the dose rate for administration of radiation, the type of radiation used, and the oxygen status of the target cells.

The last three factors were fixed in the current work. Cells were exposed to gamma rays from a ^{60}Co unit. Most experiments in the literature are conducted using x-rays or gamma rays. These two types of radiation are comparable in the production of biological damage[76]. The radiation dose rate used in the current work was a factor of 2 lower than that used in the majority of experimental studies with cisplatin and radiation[73, 92, 79, 82, 71]. It is expected that the survival curves for drug and radiation alone will have a slightly larger shoulder than would be seen with a higher radiation dose rate. This reflects the large amount of repairable radiation-induced damage produced with low radiation dose rates as opposed to the abundance of lethal damage produced with high

Experiment #	Treatment #1	Wait	Treatment #2
1	Cisplatin	10 minutes	Radiation
2	Cisplatin	60 minutes	Radiation
3	Radiation	10 minutes	Cisplatin
4	Radiation	60 minutes	Cisplatin

Table 2: Order and timing schedules for cisplatin and radiation administration.

radiation dose rates. If cisplatin can radiosensitize cells by inhibiting radiation-induced repair, then the effect of using a relatively low radiation dose rate for combination treatment, may be to increase cell kill since extensive repair occurs with low dose rate radiation[71].

No particular attention was paid to the oxygenation of cells during treatment. That is, the cells were treated in an ambient oxygen environment. The cells were therefore not hypoxic. Some experimenters have found that oxygen status is an important factor in the outcome of combination radiation-cisplatin treatment. Others have found the oxygen status unimportant. The experiments conducted for this work were done in the presence of oxygen. This was chosen as a starting point because it was much less complicated than fixing the gas concentration in the irradiated samples to create a hypoxic environment.

The three factors that were varied throughout the experiments were the radiation doses and drug concentrations administered, the timing between their administration and the order of administration. These factors have been shown to largely vary the outcome of combination treatment. The radiation doses and drug concentrations were varied so that the entire survival curves were explored. This was done in an attempt to tease out any dependency of outcome on radiation dose or drug concentration. Most experiments to date have been both arbitrary and inconsistent in their choices of radiation doses and drug concentrations to investigate. This has lead to conflicting observations as to the

efficacy of combination treatment and the doses and concentrations at which efficacy might exist. The drug concentrations and radiation doses used in combination treatment were determined by producing a survival curve and choosing concentrations and doses that resulted in surviving fractions (f) of approximately $f > 0.9$, $0.9 > f > 0.5$, $0.5 > f > 0.1$, $f < 0.1$.

Two time points were considered for administration of the second treatment relative to the first. The second treatment was given either 10 minutes or 60 minutes after the first treatment. The 10 minute time point was used to see if an effect could be induced from the second insult occurring while repair of the first insult was still in its early stages. Radiation induced double strand DNA breaks are repaired with a half-life that is bimodal. Many of these breaks are repaired with a half-life of 10 minutes, and the remaining breaks are repaired with a half-life of 60 minutes. The 60 minute time point was examined as a comparison for the 10 minute point.

Data Analysis

Chi-square Goodness of Fit

When comparing an observed survival curve to the survival curve that is expected, it is helpful to quantify the goodness of fit. The chi-square statistic, χ^2, is the ratio of the estimated variance of the fit to the variance of the sampled data, multiplied by the number of degrees of freedom, as described in equation 1.

$$(1) \qquad \chi_\nu^{\,2} = \frac{\chi^2}{\nu} = \frac{s^2}{\sigma^2}$$

The estimated variance of the fit represents the accuracy of the fit and the dispersion of the sampled data, while the variance of the sampled data represents only the dispersion of the sampled data. Equation 2 shows this mathematically.

$$(2) \qquad \chi^2 = \sum_{i=1}^{n} \frac{[y_i - y(x_i)]^2}{\sigma_i^{\,2}}$$

Where, y_i is the data point in the model for i = 1, 2, ..., n data points, and $y(x_i)$ is the data point to be fit to the model for the dame i value. The combined variance is σ_i^2 and is equal to the square of the sum of the standard errors of the mean for the model and fitted data points.

Therefore, from equation d the chi-square statistic quantifies the goodness of fit. If the fit between curves is good then there should be little or no difference between the estimated and sample variances. In this case the reduced chi-square statistic, χ^2/ν, will approximately equal 1. If the fit is poor, this value will be much greater than one[113]. The number of degrees of freedom used is equal to n-1.

The analysis was divided into three sections. The first deals with a comparison of combination treatments to radiation alone. The second is a comparison of combination treatments to drug alone. The third is a comparison of combinations to each other.

A Comparison of Combination Treatment and Radiation Treatment

The combination treatment was compared to radiation treatment alone by normalizing the combination survival data to the drug alone survival data. This corrects the combination data for the effect of the drug. These normalized values were then compared to the observed radiation survival data using a chi-square goodness of fit test.

This test was helpful in determining whether or not the survival curve for combined treatment was statistically different from the survival curve for radiation treatment alone. Figure (iii) illustrates the points being compared for each combination. Each data point on the combination curve is compared to the corresponding data point on the radiation curve. The comparison includes all the data points across the two curves.

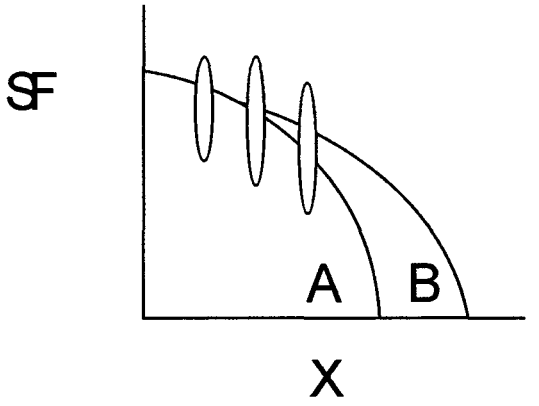


Figure (iii): Chi-square is the comparison of several pairs of data points between the combination survival curve A, and the radiation survival curve B, for radiation doses X and surviving fractions SF. The comparisons are summed over the length of the lines.

Since the variance for each data point on each curve is poorly estimated by the small sample number of three, in general the variances are large and can fluctuate greatly over the length of the survival curves. The chi-square value is a sum of the ratio of observed to expected variances along the length of the survival curve, so the relative contribution of individual pairs of data points on the curve, to a statistically significant chi-square value is not recognized. The individual chi-square values that contribute to the value returned by the chi-square test may be examined to determine which pairs of points contribute to a significant difference between the curves. If the data points along the curve are consistent in their contribution to the chi-square test value, then it can be concluded that the curves are truly different. If the chi-square test value is largely influenced by only one or two pairs of data points then there is evidence that the curves are not different, despite the result of the chi-square test. An examination of the individual chi-square values was the second tool used in determining whether or not a difference existed between the combination and radiation only curves.

A Comparison of Combination Treatment and Drug Treatment

The radiation normalized data was analyzed with the same statistical tests as in the comparison to radiation treatment. The chi-square test helped in identifying a pattern of performance for the combinations compared to drug alone. The comparisons made are illustrated in figure (iv).

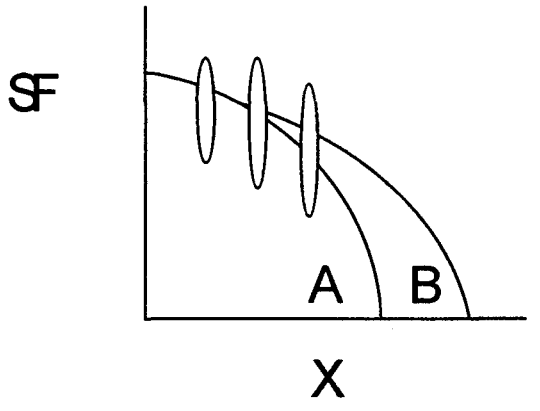


Figure (iv): Chi-square is the comparison of several pairs of data points between the combination survival curve A, and the cisplatin survival curve B, for cisplatin concentrations X and surviving fractions SF. The comparisons are summed over the length of the lines.

Two-Sample Student's t-test

The two-sample Student's t-test can be used to test for a difference between two sample means. The number of degrees of freedom for small samples is $(n_1 + n_2 - 2)$, where n_1 and n_2 are the sizes of each sample to be compared. If the test statistic is greater than or equal to the critical t value for a given level of confidence, then the hypothesis that the two means are equal is rejected. The test statistic is defined by equation 3,

$$(3) \qquad t = \frac{x_1 - x_2}{\sigma^2}$$

where, x_1 and x_2 are the means of each sample and σ^2 is the combined variance[114].

A Comparison of Combination Treatment and Radiation Treatment

A two-sample Student's t-test was used to determine whether or not there was a statistically significant difference between each combination data point and the radiation only data point at the same radiation dose. An illustration of the points being compared is given in figure (v). The comparison is for individual pairs of data points only.

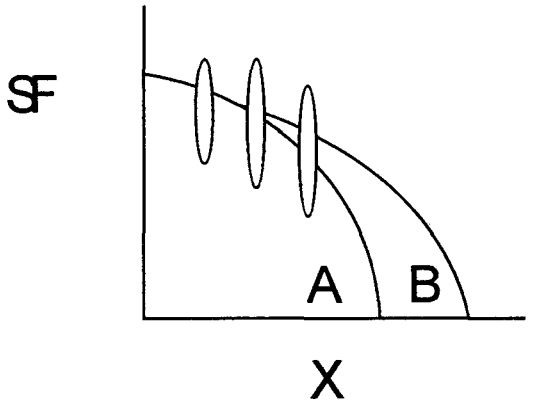


Figure (v): The t-test is a comparison between individual pairs of data points between the combination survival curve A, and the radiation survival curve B, for radiation doses X and surviving fractions SF. The t-test only compares pairs of points between two lines and not the lines themselves.

The chi-square goodness of fit test uses the square of the difference between the two means which means that information regarding the direction in which one curve deviates from the other is no longer known. Therefore, a given value for the chi-square test may represent a combination curve that is above or below the radiation survival curve, or a curve that criss-crosses the radiation survival curve. Statistically, the lines in each case are the same. However, a statistical analysis can not always be relied upon as the only method of data analysis. In the situation where the standard errors are calculated from poor estimates of the sampled population variance the chi-square and t-distributions are very broad and it is difficult to reject the null hypothesis of equal lines or equal means at the 95% confidence level. Therefore, an important part of the data analysis is the identification of patterns in the data. The calculated t values can be used to help identify patterns of response in the combination data by quantitatively differentiating between parts of the curves that deviate from the radiation curve in different directions. The t values can be examined along the length of the two survival curves. Deviations in the positive direction indicate an increase in survival relative to radiation treatment. Negative deviations indicate a decrease in survival relative to radiation. If the individual chi-square values indicate a pattern in the magnitude of the difference between the combination and radiation survival curves, the t values should be inspected to determine if the difference follows a directional pattern. An examination of the t values to determine the presence of a pattern in the deviations from the radiation only curve, is the third method of data analysis used.

A Comparison of Combination Treatment and Drug Treatment

The t-tests supported the chi-square conclusions by investigating differences between each pair of points on the two curves. The t values were also useful in determining the consistency of the pattern identified by chi-square in terms of the direction of difference. Figure (vi) shows the points that are compared for the radiation normalized data.

Analysis of Variance

The analysis of variance is similar to the chi-square goodness of fit test in that it uses a ratio of variances to determine whether or not two points are different. The analysis of variance can be used to determine if any of a number of means are the same as one another. It does this by comparing the between mean variation to the variation within each sample mean. The test statistic is given by equation 4:

$$(4) \qquad F = \frac{N\sigma^2}{\sum_{i=0}^{N} \frac{\sigma_i^2}{n}}$$

Where N is the number of samples to be compared and σ^2 is the variation between sample means. The value σ_i^2 is the variation within each sample, n is the sample size and the denominator is, therefore, an estimation of the average within sample variation[115].

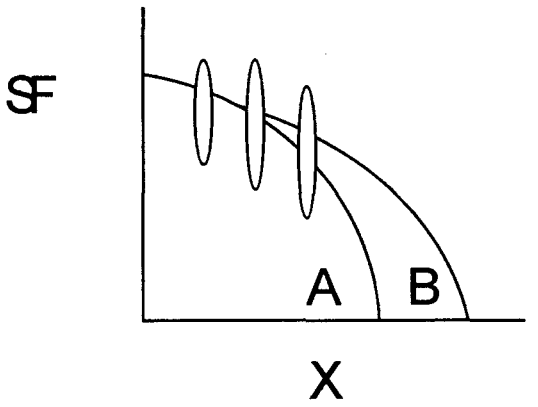


Figure (vi): The t-test is a comparison between individual pairs of data points between the combination survival curve A, and the cisplatin survival curve B, for cisplatin concentrations X and surviving fractions SF. The t-test only compares pairs of points between two lines and not the lines themselves.

A Comparison of Combination Treatments With One Another

In order to compare any two combination treatments within a schedule the combination data must be corrected for both drug and radiation effects. The combination data were therefore normalized to both drug alone and radiation alone. This analysis was effective in comparing combination treatment to other combination treatments within and between schedules. Since normalizing the data to both radiation and drug corrects for all treatment, any corrected combination data points originating from a given radiation dose and drug concentration can be compared with any other corrected combination data point. Therefore, the analysis of variance was used to determine whether any one combination performed better or worse than any other combination across both the radiation dose and drug concentration ranges. Due to the small number of samples treated with each combination it was difficult to achieve statistical significance with the analysis of variance.

A Comparison of Combination Treatments With Additivity

The different combinations were also compared by evaluating the combination response with respect to the response that would be expected for an additive interaction between cisplatin and radiation. This method of comparison depends on defining a model for additivity where deviations from additivity imply synergy or antagonism

depending on the direction of the deviation. Assuming that radiation and cisplatin are non-competitive inhibitors, the expected surviving fraction (f) for a given combination treatment in achieving additivity is given by equation 5:

$$(5) \qquad f_{comb} = f_{rad} f_{drug}$$

Upon rearranging,

$$(6) \qquad \frac{f_{comb}}{f_{rad} f_{drug}} = 1$$

Where f_{comb} is the surviving fraction for the given combination and f_{rad} and f_{drug} are the surviving fractions for radiation and drug, respectively, at the same dose and concentration used in the combination.

A combination for which the left hand side of equation 6 is less than one is then synergistic. A combination for which equation 6 is greater than 1 is antagonistic. Since normalization of the combination data for radiation and drug is achieved by dividing the combination survival data by the radiation only and drug only survival data, the twice normalized combination data can be substituted into the left hand side of equation 6 and evaluated for synergy. The chi-square goodness of fit test, an examination of individual chi-square values and an inspection of t values, were all used to compare the combination

indices for combinations, using the same radiation dose or using the same drug concentration, to 100% which represents additivity . The comparisons were done by group to see if the combination response relative to additivity depended on the radiation dose or drug concentration used. The data was also examined, using the same methods of analysis, by grouping the data according to low dose and concentration, moderate dose and concentration, and high dose and concentration.

Median Effect Analysis

Another model for synergy is given by the median-effect analysis[89]. This model describes additivity for two cases. For the case where radiation and cisplatin are non-competitive inhibitors, additivity is given for a combination index (CI) that is equal to unity. A combination whose value is less than one is synergistic, and a CI greater than one is antagonistic. The combination index is defined in equation 7.

$$(7) \qquad CI = \frac{Dc}{Do} + \frac{Rc}{Ro}$$

Here, Dc and Rc are the drug concentration and radiation dose, respectively, that are used in the particular combination treatment. Do and Ro are the drug concentration and radiation dose, respectively, that are used alone to achieve a given fraction of survival. The combination index must be evaluated at a certain survival endpoint. The

CI values are conventionally determined for 50% survival and plotted as an LD 50 (dose that kills 50%) isobologram. An isobologram is the plot of one of the quotients in equation 7 versus the other quotient. Equation 7 can be used to make isobolograms for other fractions of cell survival as well. Another common isobologram endpoint is the LD 90 (doses that kill 90%).

The two quotients that make up the combination index each incorporate some very interesting information that may be obscured by the sum. Each quotient has the ability to describe the amount that the dose or concentration was reduced by being given in combination. The general equation for the quotient, Q, of a treatment, T, given in combination described by equation 8.

$$(8) \qquad Q = \frac{Tc}{To}$$

Where Tc is the amount given in combination and To is the amount given alone for a given surviving fraction. The inverse of this equation is called the dose reduction index $(DRI)^{90}$ and is defined in equation 9.

$$(9) \qquad DRI = \frac{To}{Tc}$$

This value describes the factor by which the amount given in the treatment alone is greater than that given in the combination for the same surviving fraction. The DRI values were inspected to see if they varied consistently with radiation dose or drug concentration.

The median-effect analysis describes additivity in the situation for two competitive inhibitors by equation 10.

$$(10) \qquad CI = \frac{Dc}{Do} + \frac{Rc}{Ro} + \frac{DcRc}{DoRo}$$

Compared to the non-competitive model, this CI defines a value for additivity that is more conservative in concluding that synergy exists. Since the competitive model for additivity is based on the non-competitive model, and neither model will indicate whether or not the cisplatin-radiation interaction is competitive or not, but will merely describe the combination response for that model, and the non-competitive model is more intuitive, the data was analyzed assuming radiation and cisplatin are two non-competitive inhibitors.

Median effect analysis was used to make LD 50 and LD 90 isobolograms for the fourth experiment where drug was given 60 minutes after radiation. The DRI values were also examined to see if they indicated synergy or antagonism in a way that depended on radiation dose or drug concentration.

A Comparison of Combination Treatments Between Schedules:

In order for the combinations to be compared across schedules, the normalizing factors have to be comparable. That is, the right hand side of equation 5 must be the same for each schedule. This ensures that the combination values all stay relative to a common point of comparison, or a common value of additivity. Since the radiation and drug survival curves were consistent between each schedule, the product in the right-hand side of equation 5 is constant. Therefore, a comparison of combination treatments between schedules can be performed.

Results

Survival Curve for SCC-25 Cells and Cisplatin

The cisplatin survival curve was repeated three times for each of the four combination schedules. The survival data was averaged over the twelve resulting experiments. The means and standard errors of the means for cisplatin survival are plotted in figure 1a for concentrations ranging from 0.1 to 20 uM. Figure 1b shows the cisplatin survival over the lower cisplatin concentration of 0.1 to 5 uM. In both cases the per cent survival is plotted on a logarithmic scale. The drop lines in figures 1a and 1b identify the mean survival for each drug concentration used. Approximately 1 uM of cisplatin is required to kill 10 per cent of the cells in the sample. About 10 uM of cisplatin is needed to kill 50 per cent of the cells. Survival drops to 20 per cent at 20 uM. The concentrations 0.1, 1, 5, 10 and 20 uM correspond to the survival percentages listed in Table 3.

Concentrations of cisplatin resulting in a lethal dose to approximately 10, 50 and 90 per cent of cells were chosen for combination with radiation (1, 10 and 20 uM). Two

Cisplatin Concentration	Per Cent Survival
0.1 uM	103.82 +/- 3.19
1 uM	87.87 +/- 4.19
5 uM	75.96 +/- 2.39
10 uM	58.25 +/- 3.99
20 uM	19.77 +/- 3.77

Table 3: Percentage survival for cisplatin treatment in SCC-25 cells.

other cisplatin concentrations were chosen such that the corresponding cell kill was relatively low (0.1 and 5 uM).

Survival Curve for SCC-25 Cells and Radiation

The radiation survival curve was repeated for each of the four combination schedules. The survival data was averaged over the four experiments. The means and standard errors of the means are plotted in figure 2a for radiation doses ranging from 0.05 to 7.0 Gy. Figure 2b shows the radiation survival curve over the radiation doses of 0.05 to 1.0 Gy. In both cases the per cent survival is plotted on a logarithmic scale.

The drop lines in figures 2a and 2b identify the mean survival for each radiation dose used. Approximately 0.25 Gy is required to kill 10 per cent of the cells in the sample. About 2.5 Gy is needed to kill 50 per cent of the cells. Survival drops to just under 5 per cent at 7.0 Gy. The doses 0.05, 0.25, 1.0, 2.5 and 7.0 Gy correspond to the survival percentages listed in table 4.

Radiation doses resulting in a lethal dose to approximately 10, 50 and 90 per cent of cells were chosen for combination with cisplatin (0.25, 2.5 and 7.0 Gy). Two other radiation doses were chosen such that the corresponding cell kill was relatively low (0.05 and 1.0 Gy).

Radiation Dose	Per Cent Survival
0.05 Gy	98.30 +/- 2.34
0.25 Gy	93.08 +/- 1.95
1.0 Gy	82.49 +/- 2.78
2.5 Gy	54.11 +/- 2.31
7.0 Gy	3.65 +/- 0.61

Table 4: Percentage survival for radiation treatment in SCC-25 cells.

Effect of Cisplatin on Radiation Survival of SCC-25 Cells

The survival data for samples treated with combinations of drug and radiation were corrected for the effect of cisplatin treatment. This was accomplished by dividing the per cent survival for a given combination treatment by the surviving fraction for the corresponding concentration of cisplatin as determined from the cisplatin survival curve. All data presented are mean values for three separate experiments. Within each of the three experiments, only one sample was prepared per combination treatment. The combination survival data were normalized to cisplatin survival within each experiment and then the normalized data were averaged to get the mean values. Each mean is shown with its standard error.

Cisplatin Administration 60 Minutes Before Radiation

The effect of cisplatin on radiation survival was determined by comparing the cisplatin normalized combination survival curve to the radiation survival curve. The survival data are plotted in figures 5 and 6 as a function of radiation dose for a given concentration of cisplatin pretreatment. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with cisplatin 60 minutes before radiation results in a survival increase relative to radiation alone. The survival increase is not seen at any radiation dose when cells are pretreated with 0.1 uM (figures 5a and 6a) or for 1 uM cisplatin (figures 5b and 6b). The survival increase is first seen for 5 uM cisplatin treatment with 0.05 Gy radiation (figures 5c and 6c). The effect is much more evident for 10 uM cisplatin and 0.05, 0.25 and 1.0 Gy (figures 5d and 6d). The mean survival values for 20 uM given with 0.05 and 0.25 Gy are consistent with the survival increase, however, the standard errors are extremely large and overlap with the errors from the radiation survival curve (figures 5e and 6e).

In general, the survival increase is most prominent after treatment with 0.05 Gy radiation. Figure 7 shows the change in radiation survival as a function of the cisplatin pretreatment concentration. For radiation doses of 0.05, 0.25 and 1.0 Gy the survival appears to increase for 0.1 uM pretreatment, decrease for 1 uM and then gradually increase over the concentrations 5, 10 and 20 uM. These changes are of comparable magnitude for 0.05 and 0.25 Gy (figures 7a and 7b). For 1.0 Gy the increase over 5 to 20 uM is not as great (figure 7c). For 2.5 Gy there is no significant survival increase for any of the cisplatin concentrations used (figure 7d).

Cisplatin Administration 10 Minutes Before Radiation

The effect of cisplatin on radiation survival was determined by comparing the cisplatin normalized combination survival curve to the radiation survival curve. The survival data are plotted in figures 8 and 9 as a function of radiation dose for a given concentration of cisplatin pretreatment. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with cisplatin 10 minutes before radiation does not change radiation survival. This is true regardless of the cisplatin concentration used (figures 8 and 9).

Cisplatin Administration 10 Minutes After Radiation

The effect of cisplatin on radiation survival was determined by comparing the cisplatin normalized combination survival curve to the radiation survival curve. The survival data are plotted in figures 10 and 11 as a function of radiation dose for a given concentration of cisplatin post-treatment. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with cisplatin 10 minutes after radiation results in a survival increase relative to radiation alone. The increase is present for 0.1 uM cisplatin given after 0.25, 1.0 and 2.5 Gy (figures 10a and 11a). No consistent increase is apparent for 1 uM

combinations (figures 10b and 11b) or for 5 uM combinations (figures 10c and 11c). The mean survival values for both 10 (figures 10d and 11d) and 20 uM (figures 10e and 11e) given with 0.05 and 0.25 Gy are consistent with the survival increase, however, the standard errors are extremely large and overlap with the errors from the radiation survival curve.

The survival increase is most prominent for treatment with 0.05 Gy radiation. Figure 12 shows the change in radiation survival as a function of the cisplatin post-treatment concentration. For radiation doses of 0.05, 0.25, 1.0 and 2.5 Gy the survival appears to increase for 0.1 uM post-treatment, decrease for 1 uM and then gradually increase over the concentrations 5, 10 and 20 uM. These changes are of comparable magnitude for 0.05 and 0.25 Gy (figures 12a and 12b). For 1.0 and 2.5 Gy there is limited increase over 5 to 20 uM (figures 12c and 12d).

Cisplatin Administration 60 Minutes After Radiation

The effect of cisplatin on radiation survival was determined by comparing the cisplatin normalized combination survival curve to the radiation survival curve. The survival data are plotted in figures 13 and 14 as a function of radiation dose for a given concentration of cisplatin post-treatment. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with cisplatin 60 minutes after radiation results in a survival increase relative to radiation alone. The increase is present for 0.1 uM cisplatin given after 0.05, 0.25, and 1.0 Gy (figures 13a and 14a). An increase is also apparent for 1 uM with 0.05 and 1.0 Gy. The mean survival value for 1 uM with 0.25 Gy is consistent with a survival increase, however, the standard errors are large and overlap the radiation survival curve (figures 13b and 14b). There is no increase for 5 uM post-treatment (figures 13 c and 14 c). The mean survival value for 10 uM with 0.05 and 0.25 Gy is consistent with a survival increase, however, the standard errors are large and overlap the radiation survival curve (figures 13d and 14d). The survival increase is very large for 20 uM with 0.05, 0.25 and 1.0 Gy (figures 13e and 14e).

The survival increase is quite prominent for treatment with 0.05 Gy radiation. Figure 15 shows the change in radiation survival as a function of the cisplatin post-treatment concentration. For radiation doses of 0.05, 0.25, 1.0 and 2.5 Gy the survival appears to increase for 0.1 uM post-treatment, decrease for 5 uM and then gradually increase over the concentrations 5, 10 and 20 uM. These changes are of comparable magnitude for 0.05 and 0.25 Gy (figures 15a and15b), except at 20 uM where the survival increase for 0.05 Gy is very much larger. For 1.0 Gy the survival fluctuates considerably as cisplatin post-treatment concentration increases (figure 15c). For 2.5 Gy there is no significant increase over the cisplatin treatment range of 5 to 20 uM (figure 15d).

Effect of Radiation on Cisplatin Survival of SCC-25 Cells

The survival data for samples treated with combinations of drug and radiation were corrected for the effect of radiation treatment. This was accomplished by dividing the per cent survival for a given combination treatment by the surviving fraction for the corresponding dose of radiation as determined from the radiation survival curve. All data presented are mean values for three separate experiments. Within each of the three experiments, only one sample was prepared per combination treatment. The combination survival data were normalized to radiation survival within each experiment and then the normalized data were averaged to get the mean values. Each mean is shown with its standard error.

Radiation Administration 60 Minutes Before Cisplatin

The effect of radiation on cisplatin survival was determined by comparing the radiation normalized combination survival curve to the cisplatin survival curve. The survival data are plotted in figures 16and 17 as a function of cisplatin concentration for a given pretreatment dose of radiation. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with radiation 60 minutes before cisplatin results in a survival increase relative to cisplatin alone. The survival increase is seen for 0.05 Gy radiation with 0.1 and 1 uM cisplatin (figures 16a and 17a). The effect is also evident for 0.25 Gy given prior to 0.1, 1 and 5 uM cisplatin (figures 16b and 17b). The increase is visible for 1.0 Gy with 0.1 and 1 uM (figures 16c and 17c). For 2.5 Gy given prior to 0.1 uM, an increase also exists (figures 16d and 17d). The mean survival value for 7.0 Gy given with 0.1 uM is consistent with a survival increase, however, the standard error is extremely large and overlaps with the cisplatin survival curve (figures 16e and 17e). For 7.0 Gy combined with cisplatin concentrations greater than 1 uM, the survival decreases relative to drug alone.

The survival increase is most prominent after treatment with 0.1 uM cisplatin. Figure 18 shows the change in cisplatin survival as a function of the radiation pretreatment dose. For the 0.1 uM cisplatin concentration, the survival appears to increase dramatically for 0.05 Gy, then gradually from 0.05 Gy to 7.0 Gy (figure 18a). For cisplatin concentrations of 1, 5, 10 and 20 uM, the survival increases for radiation doses of 0.05, 0.25 and 1.0 Gy and then decreases for radiation doses up to 7.0 Gy (figures 18b to 18e).

Radiation Administration 10 Minutes Before Cisplatin

The effect of radiation on cisplatin survival was determined by comparing the radiation normalized combination survival curve to the cisplatin survival curve. The survival data are plotted in figures 19 and 20 as a function of cisplatin concentration for a given pretreatment dose of radiation. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with radiation 10 minutes before cisplatin results in a survival increase relative to cisplatin alone. There is no survival increase for 0.05 Gy (figures 19a and 20a). The survival increase is seen for 0.25 Gy radiation with 0.1 uM (figures 19b and 20b). The increase is also seen for 1.0 and 2.5 Gy with 0.1uM cisplatin (figures 19c,d and 20c,d). There is no survival increase for 7.0 Gy, regardless of the cisplatin concentration used (figures 19e and 20e).

The survival increase is most prominent after treatment with 0.1 uM cisplatin. Figure 21 shows the change in cisplatin survival as a function of the radiation pretreatment dose. For the 0.1 uM cisplatin concentration, the survival increases for radiation doses up to 1.0 Gy then gradually decreases from 1.0 to 7.0 Gy (figure 21a). For cisplatin concentrations of 1, 5, 10 and 20 uM, the increase in survival for radiation doses up to 1.0 Gy is not as large (figures 21b to 21e) and survival decreases only marginally for doses between 1.0 and 7.0 Gy.

Radiation Administration 10 Minutes After Cisplatin

The effect of radiation on cisplatin survival was determined by comparing the radiation normalized combination survival curve to the cisplatin survival curve. The survival data are plotted in figures 22 and 23 as a function of cisplatin concentration for a given post-treatment dose of radiation. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with radiation 10 minutes after cisplatin does not result in a survival increase relative to cisplatin alone (figures 22 and 23). A decrease in survival is evident for 2.5 and 7.0 Gy with both 10 and 20 uM cisplatin (figures 22d and 22e).

Radiation Administration 60 Minutes After Cisplatin

The effect of radiation on cisplatin survival was determined by comparing the radiation normalized combination survival curve to the cisplatin survival curve. The survival data are plotted in figures 24 and 25 as a function of cisplatin concentration for a given post-treatment dose of radiation. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with radiation 60 minutes after cisplatin does not result in a significant survival increase relative to cisplatin alone (figures 24 and 25). There is no decrease in survival either, except for 7.0 Gy and 20 uM (figure 24e).

Survival Curve for SCC-25/CP Cells and Cisplatin

The cisplatin survival curve for SCC-25/CP was repeated three times and the survival data was averaged over three values. The means and standard errors of the means for both the SCC-25 and SCC-25/CP cell lines are plotted in figure 3 for cisplatin concentrations ranging from 0.1 to 30 uM. The per cent survival is plotted on a logarithmic scale. From 1 uM to 20 uM the SCC-25/CP cell line is more resistant to cisplatin treatment than the SCC-25 cell line. The magnitude of this resistance ranges from approximately 1.2 times at 1 uM to almost four times at 20 uM. There does not appear to be any region of the SCC-25/CP curve that is particularly steep relative to the rest of the curve. There is, therefore, no evidence of a hypersensitive or radioresistant component to the survival curve.

Survival Curve for SCC-25/CP Cells and Radiation

The radiation survival curve for SCC-25/CP was repeated three times and the survival data was averaged over three values. The means and standard errors of the means for both the SCC-25 and SCC-25/CP cell lines are plotted in figure 4a for radiation doses ranging from 0.05 to 7.0 Gy. Figure 4b shows the radiation survival curves over the radiation doses of 0.05 to 1.0 Gy. In both cases the per cent survival is plotted on a logarithmic scale. The drop lines in figure 4a identify the mean survival for each radiation dose used. For radiation doses between zero and 2.5 Gy, there is no significant difference between the survival curves of SCC-25 and SCC-25/CP cell lines. However, the SCC-25/CP cell line is more resistant to radiation at higher doses. The magnitude of this resistance is approximately three times at 7.0 Gy. There does not appear to be any region of the SCC-25/CP curve that is particularly steep relative to the rest of the curve. There is, therefore, no evidence of a hypersensitive or radioresistant component to the survival curve.

Effect of Cisplatin on Radiation Survival of SCC-25/CP Cells

Radiation Administration 60 Minutes Before Cisplatin

The effect of cisplatin on radiation survival was determined by comparing the cisplatin normalized combination survival curve to the radiation survival curve. The survival data are plotted in figures 34a and 34b as a function of radiation dose for a given concentration of cisplatin pretreatment. In both cases the per cent survival is plotted on a logarithmic scale. Treating with cisplatin 60 minutes after radiation results in a survival decrease for both 10 and 50 uM treatments. The survival decrease is observed especially for higher doses in the 10 uM case (figure 34a) and for all radiation doses in the 50 uM case (figure 34b).

Determination of Synergy and Antagonism in SCC-25 Cells

The survival data for samples treated with combinations of drug and radiation were corrected for both the effect of cisplatin and of radiation treatment. This was accomplished by dividing the per cent survival for a given combination treatment by the surviving fraction for the corresponding concentration of cisplatin as determined from the cisplatin survival curve. This quotient was then divided by the surviving fraction for the radiation dose used in the combination. The extent of synergy or antagonism was

determined by comparing the cisplatin and radiation corrected combination survival curve to the line of additivity which is equal to 100 per cent survival. All data presented are mean values for three separate experiments. Within each of the three experiments, only one sample was prepared per combination treatment. The combination survival data were normalized to cisplatin survival, then to radiation survival within each experiment and then the normalized data were averaged to get the mean values. Each mean is plotted with its standard error.

Cisplatin Administration 60 Minutes Before Radiation

The survival data are plotted in figures 26 and 27 as a function of radiation dose for the concentration of cisplatin used in the combination. Figure 26 shows the response over the entire radiation dose range of 0.05 to 7.0 Gy. Figure 27 shows the response over the radiation dose range of 0.05 to 2.5 Gy. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with cisplatin 60 minutes before radiation results in a survival increase relative to the line of additivity. The mean survival values for 0.1 uM with 0.05 Gy (figure 27a) is consistent with the survival increase, however, the standard errors are extremely large and overlap with the line of additivity. There is a survival decrease for 1 uM of cisplatin given prior to 0.25 and 1.0 Gy (figure 27b). The combinations of 1 uM with

0.05 and 2.5 Gy also dip below the line of additivity, however, the standard errors overlap with the additivity line (figure 27b). The survival increase is first seen for 5 uM cisplatin treatment with 0.05 and 0.25 Gy radiation (figure 27c). The effect is much more evident for 10 uM cisplatin and 0.05, 0.25 and 1.0 Gy (figure 27d). The mean survival values for 20 uM given with 0.05 and 0.25 Gy (figure 27e) is consistent with the survival increase, however, the standard errors are extremely large and overlap with the line of additivity. A decrease in survival compared to the additivity line is observed for this schedule when relatively large concentrations of cisplatin and high radiation doses are used, as can be seen from figures 26c and 26e. The improved survival is increasingly prominent for increasing concentrations of cisplatin and 0.05 Gy radiation.

Cisplatin Administration 10 Minutes Before Radiation

The survival data are plotted in figures 28 and 29 as a function of radiation dose for the concentration of cisplatin used in the combination. Figure 28 shows the response over the entire radiation dose range of 0.05 to 7.0 Gy. Figure 29 shows the response over the radiation dose range of 0.05 to 2.5 Gy. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with cisplatin 10 minutes before radiation does not change radiation survival significantly at low radiation doses for any cisplatin concentration (figure 29). A

decrease in survival relative to the additivity line is seen for larger cisplatin concentrations and high doses of radiation (figures 28d and 28e).

Cisplatin Administration 10 Minutes After Radiation

The survival data are plotted in figures 30 and 31 as a function of radiation dose for the concentration of cisplatin used in the combination. Figure 30 shows the response over the entire radiation dose range of 0.05 to 7.0 Gy. Figure 31 shows the response over the radiation dose range of 0.05 to 2.5 Gy. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with cisplatin 10 minutes after radiation results in a survival increase relative to the line of additivity. The increase is present for 0.1 uM cisplatin given after 0.25, 1.0 and 2.5 Gy (figure 31a). No increase is apparent for 1 uM combinations (figures 30b and 31b) or for 5 uM combinations (figures 30c and 31c). There is a significant increase in survival for 10 uM and 0.05 Gy (figure 31d). The mean survival value for 10 uM and 0.25 Gy is consistent with the survival increase, however, the standard errors are extremely large and overlap with the line of additivity (figure 31d). A survival decrease is seen for 20 uM and 7.0 Gy radiation (figure31e). The survival increase is most prominent for treatment with small concentrations of cisplatin and low radiation doses.

Cisplatin Administration 60 Minutes After Radiation

The survival data are plotted in figures 32 and 33 as a function of radiation dose for the concentration of cisplatin used in the combination. Figure 32 shows the response over the entire radiation dose range of 0.05 to 7.0 Gy. Figure 33 shows the response over the radiation dose range of 0.05 to 2.5 Gy. In all cases the per cent survival is plotted on a logarithmic scale.

Treating with cisplatin 60 minutes after radiation results in a survival increase relative to the line of additivity. The increase is present for 0.1 uM cisplatin given after 0.05, 0.25, 1.0, and 7.0 Gy (figure 33a). An increase is also apparent for 1 uM with 0.05, 0.25 and 1.0 Gy (figure 33b). There is no consistent difference between the line of additivity and combinations involving 5 uM (figures 32c and 33c). A survival increase is also suggested by combinations with 10 uM for radiation doses of 0.25 and 1.0 Gy (figure 33d). The survival increase is very large for 20 uM with 0.05, 0.25 and 1.0 Gy (figure 33e). The survival increase is present for cisplatin concentrations ranging from 0.1 to 20 uM and radiation doses up to 2.5 Gy. The increase is quite prominent for almost all combinations. There is no significant decrease in survival for large cisplatin concentrations and high radiation doses (figures 32c to 32e).

Determination of Synergy and Antagonism in SCC-25/CP Cells

Cisplatin Administration 60 Minutes After Radiation

The survival data corrected for both the effect of cisplatin and of radiation are plotted in figures 35a and 35b as a function of radiation dose for the concentration of cisplatin used in the combination. Both figures show the response over the entire radiation dose range of 0.05 to 7.0 Gy. In both cases the per cent survival is plotted on a logarithmic scale.

Treating with cisplatin 60 minutes after radiation results in a survival decrease relative to the line of additivity. A decrease is suggested for 10 uM with 0.05 and 7.0 Gy, although the standard errors are large for these points (figure 35a). The decrease is very apparent for 50 uM cisplatin given after each radiation dose used (figure 35b).

Median Effect Analysis for SCC-25 Cells

The response to combination cisplatin-radiation treatment for cisplatin given 60 after radiation was analyzed by fitting all of the survival curves to the median effect equation and producing isobolograms and DRI values.

Plotting the data on an isobologram can be advantageous because the isobologram describes the combination response relative to additivity for a given survival endpoint. The LD 50 isobologram shows a general antagonism for combinations of cisplatin and radiation that kill 50% of cells. The plot is illustrated in figure 36a. In defining the expected line of additivity, the isobologram assumes that the survival curves for the two agents in combination are linear. When the shape of either curve is not linear, the additivity line must be modified based on the shape of each survival curve. The survival curves of radiation and cisplatin have similar shapes for surviving fractions ranging from 1.0 to 0.5. Therefore, the modified and expected additivity lines for the LD 50 isobologram are approximately the same, with the exception of one stray point. The LD 90 isobologram shows antagonism as well, relative to the expected additivity line. However, the combinations are less antagonistic, and one combination even appears synergistic, if the line of additivity is modified. The isobologram appears in figure 36b.

The DRI values for drug and radiation were calculated and plotted as a function of treatment in figure 37a. The DRI value for cisplatin describes the factor by which the combination treatment has decreased the concentration given, relative to when cisplatin is administered alone. Therefore, the aim is to maximize the DRI value for a given combination treatment. It can be seen that for low drug concentrations and low radiation doses, the DRI values are small and approaching zero so the effect of combination treatment is poor relative to cisplatin alone. The DRI values for 2.5 Gy are relatively large

and the values for 7.0 Gy are extremely large and off-scale. In general, for high radiation doses, the DRI for cisplatin is very high. This implies that combination treatment with high radiation doses is beneficial in reducing the amount of cisplatin used. This is consistent with the previous finding that antagonism occurs for combinations with low radiation doses.

The DRI values for radiation are plotted in figure 37b. All combinations with 0.05 and 2.5 Gy are off-scale. It can be seen that for higher radiation doses and low drug concentrations, the radiation DRI values are less than one and approaching zero. This suggests that combinations with high drug concentrations and low radiation doses are more effective in reducing the amount of radiation used. In general, it seems that the combinations that are more effective in cell killing, are those with higher concentrations of cisplatin. The outcome of combination treatment does not appear to depend as consistently on the radiation dose used, as it does on the drug concentration.

Statistical Analysis for SCC-25 Cells

Cisplatin Administration 60 Minutes Before Radiation

The drug normalized combination survival curves for each drug concentration were compared to the radiation survival curve using the chi-square test. The critical chi-square values that must be equaled or exceeded in order to reject the null hypothesis that the survival curves are the same, are listed in table 5. Upper and lower critical values are given for a two-tailed test with 95% and 90% confidence levels.

chi-square	Two-tailed	4 df
95%	Upper Alpha/2	11.143
	Lower Alpha/2	0.484
90%	Upper Alpha/2	9.488
	Lower Alpha/2	0.711

Table 5: Critical chi-square values for 4 degrees of freedom.

The values depend on the number of degrees of freedom for the chi-square test which is determined by the number of points in the curves being compared. All five radiation doses, 5, 25, 100, 250 and 700 rad, were included in the determination of the chi-square values. The corresponding number of degrees of freedom for this test is 4. Table 6 shows the calculated chi-square values.

chi-square	4 df
0.1 μM	0.847
1 μM	3.644
5 μM	5.527
10 μM	**38.096**
20 μM	2.971

Table 6: Calculated chi-square values for 4 degrees of freedom.

None of the chi-square values calculated meet or exceed the critical chi-square values, except the values for the combination line at 10 uM at 95%. The entire line appears to differ significantly from the radiation curve by this analysis. The t-test values for all points on the curve should be examined to determine if the curve is genuinely different or if a single pair of data points is influencing the result of the chi-square test.

Each combination survival curve was looked at in more detail for deviations from the radiation survival curve. Each data point on the combination survival curve was compared to the radiation survival curve at the same radiation dose using a two-sample Student's t-test. The critical values for a two-tailed test at the 95% and 90% confidence levels are listed in table 7. The number of degrees of freedom for a test comparing two samples each of size n=3, is 4. For cases in which one of the samples did no reach the end of the assay, the sample size is n=2 and the number of degrees of freedom is 2.

t-test	4 df	2 df
95%	2.776	4.303
90%	2.132	2.920

Table 7: Critical t-test values for 4 and 2 degrees of freedom.

For combinations involving 250 and 700 rad, only two samples were used for analysis due to the third sample's failure to complete the assay. None of the t-test values for either of these curves were found to be significant when compared to the radiation survival curve. The calculated t-test values are in table 8.

t-test	5 rad	25 rad	100 rad	250 rad	700 rad
0.1 uM	0.757	0.458	0.098	0.166	0.169
1 uM	-0.515	-1.044	-1.410	-0.487	-0.246
5 uM	1.902	0.607	-1.116	-0.286	-0.462
10 uM	1.981	**5.617**	1.505	-0.592	-0.080
20 uM	0.952	0.663	0.058	-0.648	-1.096

Table 8: Calculated t-test values.

One pair of data points was found to be significant at the 95% level in the 10 uM curve. This occurred for 25 rad and 10 uM. The individual chi-square values were examined to see if it could be this point that leads to statistical difference for the 10 uM line in the chi-square test. The value for 25 rad and 10 uM was found to be 31.550 which when subtracted from the total line chi-square value of 38.096 leaves 6.546 for the line which is not significant for 3 degrees of freedom at either of the 95% or 90% confidence levels. None of the other t-test values were significant. Therefore, it is concluded that none of the combination curves are more or less effective in cell death than radiation alone.

The radiation normalized combination survival curves for each radiation dose were compared to the drug survival curve using the chi-square test. The critical chi-square values that must be equaled or exceeded in order to reject the null hypothesis that the survival curves are given in table 5. The calculated chi-square values are in table 9.

chi-square	4 df
5 rad	5.764
25 rad	5.199
100 rad	5.241
250 rad	**9.556**
700 rad	**15.204**

Table 9: Critical t-test values for 4 and 2 degrees of freedom.

None of the chi-square values calculated meet or exceed the critical chi-square values except for the 250 rad and 700 rad curves. By investigating the individual chi-square values, the dose range over which the two survival curves agree can be determined. For the 700 rad curve, significance no longer exists if the 20 uM point is excluded. The 20 uM point is therefore the influence in the significant chi-square result. For the 250 rad curve, the chi-square value at 5 uM was 7.814 which when subtracted from the summed chi-square of 9.556, gives 1.742 which is not significant for 3 degrees of freedom. These conclusions will be confirmed with a t-test.

The two-sample Student's t-test was used to confirm the results of the chi-square test for the 250 and 700 rad curves. The calculated values are listed in the table below. For 700 rad, the only point of statistical significance for 2 degrees of freedom is at 20 uM

which is the point that was already recognized as having influenced the chi-square value. For the 250 rad curve, the point at 5 uM was found to be close to statistical significance. Therefore, it is only the 5 uM point that is causing the chi-square test to return a large value. This is consistent with the individual chi-square values. The t values for all other combinations were insignificant, as indicated in table 10. Therefore, all combination treatments have the same efficacy as the drug treatment for a given drug concentration.

t-test	0.1 uM	1 uM	5 uM	10 uM	20 uM
5 rad	1.011	-0.275	1.759	1.018	0.731
25 rad	0.661	-1.373	1.01	1.341	0.241
100 rad	0.071	-1.867	-1.1389	0.631	0.236
250 rad	0.797	-0.986	**2.795**	-0.369	0.007
700 rad	1.221	0.755	0.003	1.045	**-3.4717**

Table 10: Calculated t-test values.

In order to compare combination treatments over the entire radiation dose range and drug concentration range, the survival data was normalized to both radiation and drug data. The corrected combination data points were then compared for a given radiation dose and also for a given drug concentration to identify any patterns in combination response. Corrected data points were first grouped by drug concentration and compared using an analysis of variance. The F values and corresponding probabilities are listed in table 11.

F-test	0.1 μM	1 μM	5μM	10 μM	20 μM
F	1.869	0.935	1.716	0.687	0.734
P(F)	0.209	0.490	0.238	0.621	0.593

Table 11: Calculated t-test values.

None of the F values were significant. This means that the combination response was the same for a given drug concentration despite the radiation dose used.

The corrected data points were also grouped according to radiation dose. As can be seen from the F values shown in table 12, none of the points were found to be statistically different at the 99% or 95% level.

F-test	5 rad	25 rad	100 rad	250 rad	700 rad
F	0.647	0.594	0.101	0.417	1.199
P(F)	0.669	0.705	0.989	0.826	0.376

Table 12: Calculated F-test values and corresponding probabilities.

It is difficult to discuss the relative roles of radiation and drug in producing cell death in combination, as almost all of the combination treatments produced the same surviving fraction within error.

The combination data corrected for drug and radiation were compared to the value predicted by the additivity model of 100%. The chi-square test was performed comparing the combination survival curves to a straight line through 100% survival. The

data was first grouped and compared according to drug concentration then according to radiation dose. These values and the values for each individual point are listed in table 13. The advantage of examining the chi-square for each point is that it helps to identify which points in a combination survival curve agree closely with the additivity model and which values deviate from it.

chi-square	5 rad	25 rad	100 rad	250 rad	700 rad	Row Sum
0.1uM	0.732	**2.491**	0.930	0.188	1.320	5.663
1 uM	0.074	**1.872**	3.435	1.057	0.451	6.889
5 uM	**5.381**	**2.102**	**2.051**	**90.625**	0.214	**100.376**
10 uM	2.707	**28.701**	1.412	0.114	0.652	**33.588**
20 uM	0.817	0.486	0.0002	0.824	0	2.129
Col Sum	**9.713**	**35.655**	**7.830**	**92.810**	2.638	

Table 13: Calculated chi-square values.

From table 13, the data points originating from a drug concentration of 5 uM form a line that is statistically different from the 100% line at a confidence level of 95%. The individual chi-square values suggest that the point at 250 rad is highly influential on the chi-square test while the point at0.05 Gy is large, but less extreme. The 5 uM line is still significantly different from the 100% line if the point at 250 rad is excluded. This suggests that the line may be genuinely different from 100% at each point. The t-tests will have to be explored to determine if this is true. The line of points originating from 10 uM is also statistically significant at the 95% level of confidence. The point at 25 rad seems to be the only point influencing the chi-square test towards a significant value. The majority of points agree with the 100% line. For the data grouped by radiation dose, the lines originating from 5, 25, 100 and 250 rad are significantly different from the

100% line with confidence levels of 95%, 95%, 90% and 95% , respectively. Upon inspection of the individual chi-square values it can be seen that the point at 25 rad and 10 uM is largely responsible for the significance of the 25 rad line. However, the points at 0.1 and 1 uM are sizable and therefore, the curve for 25 rad seems to differ consistently from 100% for increasing drug concentration. The points on the 100 rad curve at 1 and 5 uM contribute to the significance of the 100 rad line. Before concluding that these radiation lines are different from the 100% line, the t values should be checked.

Table 14 shows t-test values resulting from a comparison between each combination data point and the expected additivity value of 100%. The t values confirm the statistical significance of the point at 5 uM and 250 rad (95%), as well as the point at 10 uM and 25 rad (95%), which both had large individual chi-square values. There is only one other point that is statistically significant and that is the point at 5 uM and 5 rad which is significant for 90% confidence. The 5 and 100 rad lines do not consistently deviate in terms of direction from 100%. There is no consistent direction of deviation from 100% along any of the lines so there is no consistent pattern in the values that tends towards synergy of antagonism, regardless of significance. Therefore, although some combination points are different from the 100% line, in general all combination points achieve cell kill in an additive way.

t-test	5 rad	25 rad	100 rad	250 rad	700 rad	Row Sum
0.1uM	0.855	1.578	0.964	0.433	1.149	4.981
1uM	-0.272	-1.368	-1.853	-1.028	0.671	-3.850
5 uM	**2.319**	1.449	-1.432	**-9.519**	-0.462	-7.645
10 uM	1.645	**5.357**	1.188	-0.337	0.807	8.661
20 uM	0.904	0.697	0.016	-0.908	---	0.710
Col Sum	5.453	7.715	-1.115	-11.360	2.16	

Table 14: Calculated t-test values.

The individual chi-square values were also summed over groups other than the lines associated with a given drug concentration or radiation dose. To see if there is any pattern in the data that is associated with low drug concentration and low radiation dose, in general, the chi-square values were summed for combinations including the two lowest drug concentrations combined with the two lowest radiation doses. Notice that these combinations define a 2 by 2 group of four in the upper left-hand corner of the chi-square table above. Chi-square values for the three lowest drug concentrations with the three lowest radiation doses were also summed and comprise a group of nine values starting at the upper left-hand side of the above table. Similarly, the values were summed over a group of sixteen which included all values except combinations with 20 uM drug and 700 rad radiation. Finally, the chi-square values were summed over the entire treatment range of 25 values. Table 15 shows the results and the critical chi-square values for rejection of the null hypothesis that the data differs from the value 100% at confidence levels of 95% and 90%.

chi-square		Sum of 4	Sum of 9	Sum of 16	Sum of 25
		5.170	19.072	143.879	148.647
95%	Alpha/2 Upper	9.348	17.535	27.488	39.364
	Alpha/2 Lower	0.216	2.180	6.262	12.401
90%	Alpha/2 Upper	7.815	15.507	24.996	36.415
	Alpha/2 Lower	0.352	2.733	7.261	13.848

Table 15: Critical chi-square values.

All groups except the sum of four are significantly different from 100% at the 95% level. This implies that a difference exists between additivity and combination treatments over all of the treatment range except at low radiation doses and drug concentrations. However, it has already been established that the point at 5 uM and 250 rad as well as the point at 10 uM and 25 rad are the reasons for a significant chi-square. Therefore, the chi-square sum of 16 should be greatly influenced and appear significant when perhaps it is not. If the two suspected points are not included in the chi-square test then the test is only significant at the 90% level where the critical chi-square value is 22.362. This is still good evidence that some of the values contained within the group of 16 are significantly different from 100%. The sum of 25 values is no longer statistically significant if the two suspected values are removed from the sum. This is in part due to the fact that at 700 rad the variation from 100% is particularly small so the sum is not significant for the greater sample number. Therefore, there is a difference between 100% and the survival achieved by some combination treatments. There does not seem to be any pattern that can be generalized over the drug concentrations and radiation doses of the entire treatment range.

Cisplatin Administration 10 Minutes Before Radiation

The drug normalized combination survival curves for each drug concentration were compared to the radiation survival curve using the chi-square test. The critical chi-square values that must be equaled or exceeded in order to reject the null hypothesis that the survival curves are the same, are listed in table 16. Upper and lower critical values are given for a two-tailed test with 95% and 90% confidence levels.

Chi-square	two-tailed	4 df
95%	upper alpha/2	11.143
	lower alpha/2	0.484
90%	upper alpha/2	9.488
	lower alpha/2	0.711

Table 16: Critical chi-square values.

The values depend on the number of degrees of freedom for the chi-square test which is determined by the number of points in the curves being compared. All five radiation doses, 5, 25, 100, 250 and 700 rad, were included in the determination of the chi-square values. The corresponding number of degrees of freedom for this test is 4. Table 17 shows the critical values.

chi-square	4 df
0.1 μM	1.751
1 μM	1.039
5 μM	1.609
10 μM	6.017
20 μM	7.358

Table 17: Calculated chi-square values.

None of the chi-square values calculated meet or exceed the critical chi-square values in the table above. Statistically, none of the combination survival curves are different from the survival curve for radiation alone. Therefore, there is no statistical evidence or indication of a pattern to suggest that any of the combination treatments examined achieve more or less tumor cell death than radiation alone.

Each combination survival curve was looked at in more detail for deviations from the radiation survival curve. Each data point on the combination survival curve was compared to the radiation survival curve at the same radiation dose using a two-sample Student's t-test. The critical values for a two-tailed test at the 95% and 90% confidence levels are listed in table 18. The number of degrees of freedom for a test comparing two samples each of size n=3, is 4.

t-test	4 df
95%	2.776
90%	2.132

Table 18: Critical t-test values.

None of the data points on the combination survival curves were statistically significant compared to the radiation survival curve using the two-sample Student's t-test at the 90% and 95% confidence levels. There is, therefore, no statistical evidence that any of the combination treatments perform better or worse cells than radiation alone in killing tumor cells. The t values are listed in table 19.

t-test	5 rad	25 rad	100 rad	250 rad	700 rad
0.1 uM	-0.999	-0.651	0.255	0.413	0.306
1 uM	-0.445	-0.548	-0.6198	-0.3241	0.228
5 uM	0.284	1.05	0.298	0.583	-0.0005
10 uM	-1.96	-0.945	-0.2975	-0.4631	-0.9906
20 uM	-0.498	1.82	0.129	-0.0555	-1.9414

Table 19: Calculated t-test values.

The radiation normalized combination survival curves for each radiation dose were compared to the drug survival curve using the chi-square test. The critical chi-square values that must be equaled or exceeded in order to reject the null hypothesis that the survival curves are the same, are the same as previously used. Upper and lower critical values are given for a two-tailed test with 95% and 90% confidence levels in table 20.

chi-square	all data	all but 20 μM	all but 10 & 20μM
5 rad	1.612	1.611	0.922
25 rad	3.518	3.441	3.305
100 rad	1.176	1.021	1.016
250 rad	7.200	5.297	1.001
700 rad	**55.751**	**38.603**	0.947

Table 20: Calculated chi-square values.

None of the chi-square values calculated meet or exceed the critical chi-square values except at 700 rad for combination survival curves including 10 and 20 uM. By investigating the chi-square values which do not include the 20 μM points or the 20 and 10 μM points, the dose range over which the two survival curves agree can be determined. The chi-square value is significant at the 95% level for the curves that include 10 and 20 μM. The individual chi-square values for the pair of data at 10 μM is 37.655 and at 20 μM is 17.148. Therefore, curves that include either of the 10 or 20 μM points are statistically different by the chi-square test. The survival data was examined to determine why these two points might appear significant. The 10 μM point on the combination curve is almost a factor of six smaller than the radiation only point. The error associated with the combination point is not unusually large, so the chi-square returns a large value. The 20 μM point is zero. No colonies were counted in the sample and so the chi-square again registers a large value. Statistically, the 700 rad combination survival curve is the only curve that is different from the drug survival curve. However, it is worth noting that the individual chi-square values do not contribute to the large chi-square test value over the entire range of drug concentration. There is no statistical difference between the two curves over the drug concentration range 0.1 to 5 μM.

There is, therefore, some statistical evidence to suggest that of all the combination treatments examined, only the combinations involving 10 or 20 μM and 700 rad result in more tumor cell death than drug alone. This may be due to only one or two pairs of data points along the curves. This should be further investigated by looking at some t-test values for the individual pairs of data.

The two-sample Student's t-test was used to confirm the results of the chi-square test for 10 and 20 μM and various radiation doses. The calculated values are listed in the table below. The values for both 10 and 20 μM at 700 rad are significant at the 95% level. The values at 250 rad are not significant but are approaching the critical value of 2.132 for 90% confidence. The t values for all other combinations were insignificant. These results agree with the chi-square test that all combination treatments have the same efficacy as the drug treatment for a given drug concentration. There is a suggestion that for high drug concentrations and radiation doses, a difference exists between the combination and radiation only curves. The t values appear in table 21.

t-test	0.1 uM	1 uM	5 uM	10 uM	20 uM
5 rad	-0.343	-0.873	0.209	-0.83	-0.032
25 rad	-0.686	-0.708	1.528	-0.369	0.276
100 rad	0.27	-0.906	0.351	-0.07	-0.394
250 rad	-0.753	-0.583	0.309	**-2.073**	-1.38
700 rad	-0.94	0.196	-0.1607	**-6.136**	**-4.141**

Table 21: Calculated t-test values.

In order to compare combination treatments over the entire radiation dose range and drug concentration range, the survival data was normalized to both radiation and drug

data. The corrected combination data points were then compared for a given radiation dose and also for a given drug dose to identify any patterns in combination response. Corrected data points were first grouped by drug concentration and compared using an analysis of variance. The F values and corresponding probabilities are listed in table 22.

F-test	0.1 μM	1 μM	5μM	10 μM	20 μM
F	0.629	0.112	0.9953	**9.094**	0.038
P(F)	0.682	0.986	0.467	0.001	0.998

Table 22: Calculated F-test values.

Only the F value for data points compared at 10 uM was statistically significant. This means that at least two of the data points in this group are significantly different at the 0.1% level. From the survival curve of the twice normalized, it is obvious that the points at 250 and 700 rad are both candidates for being the significantly different data points.

The corrected data points were also grouped according to radiation dose. As can be seen from the F values shown in table 23, none of the points were found to be statistically different at the 99% or 95% level.

F-test	5rad	25rad	100rad	250rad	700rad
F	0.225	1.683	0.084	0.087	1.438
P(F)	0.943	0.225	0.993	0.992	0.291

Table 23: Calculated F-test values.

It is difficult to discuss the relative roles of radiation and drug in producing cell death in combination, as almost all of the combination treatments produced the same surviving fraction within error. Even for the points that are statistically different, there is no pattern in the response.

The combination data corrected for drug and radiation were compared to the value predicted by the additivity model of 100%. The chi-square test was performed comparing the combination survival curves to a straight line through 100% survival. The data was first grouped and compared according to drug concentration then according to radiation dose. These values and the values for each individual point are listed in table 24. The advantage of examining the chi-square for each point is that it helps to identify which points in a combination survival curve agree closely with the additivity model and which values deviate from it.

chi-square	5 rad	25 rad	100 rad	250 rad	700 rad	Row Sum
0.1uM	**2.183**	0.382	0.201	**13.47**	**6.202**	**22.44**
1 uM	0.185	0.634	0.160	0.202	0.391	1.574
5 uM	0.518	1.737	0.122	0.101	0.536	3.016
10 uM	**7.731**	**11.86**	0.039	**2.070**	**49.90**	**71.61**
20 uM	0.337	2.897	0.022	6.66E-05	0	3.258
Col Sum	**10.957**	**17.51**	0.547	**15.84**	**57.03**	101.9

Table 24: Calculated chi-square values.

From table 24, the data points originating from a drug concentration of 0.1 uM form a line that is statistically different from the 100% line at a confidence level of 95%. The individual chi-square values suggest that the points at 25 and 100 rad agree with the

line of additivity. The majority of the points, however, deviate from additivity which implies that it is reasonable to conclude a difference exists. This will be verified in the next paragraph by a one-sample Student's t-test. The line of points originating from 10 uM is also statistically significant at this level of confidence. The point at 100 rad seems to agree closely with the additivity model. Again, though, the majority of points do not agree with the 100% line and it can safely be said that the lines are different. For the data grouped by radiation dose, the lines originating from 5, 25, 250 and 700 rad are significantly different from the 100% line with confidence levels of 90%, 95%, 95% and 95% , respectively. Upon inspection of the individual chi-square values, however, it can be seen that only a couple of points in each radiation dose group has an unusually large deviation from the line of 100%. Its looks like the chi-square values for the drug concentrations of 0.1 and 10 uM are largely influencing the total chi-square value for each radiation line. Before concluding that the radiation lines are different from the 100% line, the t values should be checked.

The individual chi-square values were also summed over groups other than the lines associated with a given drug concentration or radiation dose. To see if there is any pattern in the data that is associated with low drug concentration and low radiation dose, the chi-square values were summed for combinations including the two lowest drug concentrations combined with the two lowest radiation doses. Notice that these combinations define a 2 by 2 group of four in the upper left-hand corner of the chi-square table above. Chi-square values for the three lowest drug concentrations combined with the three lowest radiation doses were also summed and comprise a 3 by 3 group of nine values starting at the upper left-hand side of the above table. Similarly, the values were summed over a group of sixteen which included all values except combinations with 20

uM drug and 700 rad radiation. Finally, the chi-square values were summed over the entire treatment range of 25 values. Table 25 shows the results and the critical chi-square values for rejection of the null hypothesis that the data differs from the value 100% at confidence levels of 95% and 90%.

chi-square		Sum of 4	Sum of 9	Sum of 16	Sum of 25
		3.386	6.127	41.61	101.9
95%	alpha/2 upper	9.348	17.535	27.488	39.364
	alpha/2 lower	0.216	2.180	6.262	12.401
90%	alpha/2 upper	7.815	15.507	24.996	36.415
	alpha/2 lower	0.352	2.733	7.261	13.848

Table 25: Critical chi-square values.

The only groups that are significantly different from 100% in a statistical sense are the sum of sixteen and of twenty-five. This implies that a difference exists between additivity and combination treatments that involve higher radiation doses and drug concentrations. However, it has already been determined that the line of combinations involving 250 rad has an artificially high chi-square caused by the individual chi-square at 0.1 uM. The individual chi-square values for the line of combinations including 10 uM, however, have been consistently large. Therefore, there does not seem to be any pattern that can be generalized for the entire treatment range. It still remains to be seen whether or not the points in the 0.1 uM and 10 uM lines are significantly different in a statistical sense. If a difference does exists, the direction of the difference, towards

synergy or antagonism, is still unknown. Looking at the t-test values may provide some more information.

Table 26 shows t-test values resulting from a comparison between each combination data point and the expected additivity value of 100%. It can be seen that only the 0.1 uM data points at 250 and 700 rad are significantly different from 100%. They are significant at the 95% and 90% confidence level, respectively. As well, the direction of deviation from 100% along the 0.1 uM line changes from value to value, so there is no consistent pattern in the values that tends towards synergy of antagonism. The 10 uM line has three points that are significantly different from 100%. The points at 5, 25 and 700 rad are statistically different at the 95% confidence level. The direction of deviation of the data points from 100% is consistent along the 10 uM line, even for the points that are not statistically significant. Negative values in the table represent a decrease in survival for the combination relative to the additivity line. Therefore, the values vary in the direction of synergy.

t-test	5 rad	25 rad	100 rad	250 rad	700 rad	Row Sum
0.1uM	-1.477	-0.618	0.449	**3.670**	**-2.490**	-0.466
1uM	-0.431	-0.796	-0.401	-0.449	-0.625	-2.704
5 uM	0.720	1.318	0.350	0.319	-0.732	1.975
10 uM	**-2.780**	**-3.444**	-0.197	-1.438	**-7.064**	-14.926
20 uM	-0.581	1.702	0.151	-0.008	0	1.263
Col Sum	-4.550	-1.839	0.351	2.092	-10.91	-14.85

Table 26: Calculated t-test values.

Radiation Administration 10 Minutes Before Cisplatin

The drug normalized combination survival curves for each drug concentration were compared to the radiation survival curve using the chi-square test. The critical chi-square values that must be equaled or exceeded in order to reject the null hypothesis that the survival curves are the same, are listed in table 27. Upper and lower critical values are given for a two-tailed test with 95% and 90% confidence levels.

chi-square	two-tailed	4 df
95%	upper alpha/2	11.143
	lower alpha/2	0.484
90%	upper alpha/2	9.488
	lower alpha/2	0.711

Table 27: Critical chi-square values.

The values depend on the number of degrees of freedom for the chi-square test which is determined by the number of points in the curves being compared. All five radiation doses, 5, 25, 100, 250 and 700 rad, were included in the determination of the chi-square values. The corresponding number of degrees of freedom for this test is 4. Values are shown in table 28.

chi-square	4 df
0.1 μM	**15.338**
1 μM	4.303
5 μM	7.056
10 μM	3.239
20 μM	**9.556**

Table 28: Calculated chi-square values.

The chi-square value for the curve at 0.1 uM exceeds the critical chi-square value in the table above. Most of the data points on the line differ significantly from the radiation curve by this analysis. The individual chi-square and t-test values for all points on the curve should be examined to determine if the curve is genuinely different or if a single pair of data points at lower radiation doses is influencing the result of the chi-square test. The 1 and 10 uM curves have substantial chi-square values but they are not significant at 95% or 90%. They will also be inspected in more detail with chi-square and t-tests. The 5 uM curve has a relatively large chi-square value, although it is not significant at 95% or 90%. The 20 uM line is significant at the 90% level for all data points. The individual chi-square values should again be examined before concluding that the curves are indeed different.

Each combination survival curve was looked at in more detail for deviations from the radiation survival curve. Each data point on the combination survival curve was compared to the radiation survival curve at the same radiation dose using a two-sample Student's t-test. The critical values for a two-tailed test at the 95% and 90% confidence levels are listed in table 29. The number of degrees of freedom for a test comparing two

samples each of size n=3, is 4. For cases in which one of the samples did no reach the end of the assay, the sample size is n=2 and the number of degrees of freedom is 2.

t-test	4 df	2 df
95%	2.776	4.303
90%	2.132	2.920

Table 29: Critical t-test values.

For combinations involving 5, 25, 100 rad at 1 uM, only two samples were used for analysis due to the third sample's failure to complete the assay. None of these t-test values for either of these curves were found to be significant when compared to the radiation survival curve. Values for the t-test appear in table 30.

t-test	5 rad	25 rad	100 rad	250 rad	700 rad
0.1uM	0.569	**2.936**	1.894	1.510	-0.717
1uM	-1.173	0.153	0.364	1.581	-0.517
5 uM	-0.258	0.907	-0.121	**-2.410**	-0.583
10 uM	1.380	0.691	-0.445	-0.216	-0.781
20 uM	0.745	**2.239**	-0.020	-0.953	-1.752

Table 30: Calculated t-test values.

For samples of size 3, three points registered as significant by the t-test. The point at 25 rad in the 0.1 uM line was significant at 95%. The individual chi-square values were examined to see if only one point was responsible for the significant chi-square. For 0.1 uM, both the 25 and 100 rad points have substantial individual chi-square values, however, the 5 rad point does not. This strongly suggests that the entire curve at

0.1 uM is consistent with a pattern of increased survival relative to the radiation only curve. The point at 250 rad in the 5 uM curve was significant at 90%. The individual chi-square values show that this point accounts for the large chi-square test value. The point at 25 rad in the 20 uM line was significant at 90%. However, there is no pattern evident for the 20 uM curve. Therefore, it is concluded that the combinations involving 0.1 uM could be less effective in cell death than radiation alone.

The radiation normalized combination survival curves for each radiation dose were compared to the drug survival curve using the chi-square test. The critical chi-square values that must be equaled or exceeded in order to reject the null hypothesis that the survival curves are the same, are listed in table 31. Upper and lower critical values are given for a two-tailed test with 95% and 90% confidence levels.

chi-square	4 df
5 rad	4.568
25 rad	**15.723**
100 rad	2.500
250 rad	**11.723**
700 rad	5.048

Table 31: Calculated chi-square values.

The chi-square tests show that the curve for 25 rad is statistically different from the drug survival curve at the 95% level. This suggest that the entire drug concentration range for 25 rad may be different from drug alone. This will be further investigated by the individual chi-square and t-test values. The curves for 5 and 100 rad have moderate values for the chi-square tests. The values could easily be influenced by one or two data

points along the curves. This will also be further investigated. The 250 rad survival curve is statistically significant for all data included in the chi-square test.

The two-sample Student's t-test was used to confirm the results of the chi-square test for the 25 rad curve. The calculated values are listed in the table below. The only point of statistical significance (95%) for 25 rad was at 0.1 uM which can be suspected, therefore, as the point that has influenced the chi-square value. Indeed, upon inspection of the individual chi-square values, the 0.1 uM value is unusually large. No pattern in direction of deviation from the drug only curve can be seen from the t values for 25 rad. For the 250 rad curve, the points at 0.1 uM and 20uM were significant at the 90% level. There is, however, no apparent pattern of differences with respect to the direction of deviation over the 250 rad line. The significant point at 0.1 uM looks like it belongs to the pattern of difference already identified for the 0.1 uM curve. The t values for all other combinations were insignificant, as shown in table 32. Therefore, at a given drug concentration, some combination treatments have a different efficacy than the drug treatment alone.

t-test	0.1 uM	1 uM	5 uM	10 uM	20 uM
5 rad	0.868	-0.561	-0.292	1.738	0.626
25 rad	**3.378**	0.125	0.394	0.879	1.834
100 rad	1.442	0.284	-0.057	-0.567	-0.112
250 rad	**2.257**	-0.548	-0.310	1.022	**2.277**
700 rad	-1.760	-1.341	-0.365	-0.105	-0.072

Table 32: Calculated t-test values.

In order to compare combination treatments over the entire radiation dose range and drug concentration range, the survival data was normalized to both radiation and drug data. The corrected combination data points were then compared for a given radiation dose and also for a given drug concentration to identify any patterns in combination response. Corrected data points were first grouped by drug concentration and compared using an analysis of variance. The F values and corresponding probabilities are listed in table 33.

F-test	0.1 μM	1 μM	5μM	10 μM	20 μM
F	1.869	0.935	1.716	0.687	0.734
P(F)	0.209	0.490	0.238	0.621	0.593

Table 33: Calculated F-test values.

None of the F values were significant. This means that the combination response was the same for a given drug concentration despite the radiation dose used. Therefore, it is concluded that the combination with 0.1 uM that is significantly different as identified by the previous F-test is due to the radiation dose used.

The corrected data points were also grouped according to radiation dose. As can be seen from the F values shown in table 34, the only point of statistical significance is for the curve with 5 rad. This means that at least one combination with 5rads is statistically different at the 95% level from another combination with 25 rad.

F-test	5 rad	25 rad	100 rad	250 rad	700 rad
F	**3.343**	0.261	0.312	0.709	1.61
P(F)	0.055	0.893	0.863	0.603	0.244

Table 34: Calculated F-test values.

It is difficult to discuss the relative roles of radiation and drug in producing cell death in combination, as almost all of the combination treatments produced the same surviving fraction within error.

The combination data corrected for drug and radiation were compared to the value predicted by the additivity model of 100%. The chi-square test was performed comparing the combination survival curves to a straight line through 100% survival. The data was first grouped and compared according to drug concentration then according to radiation dose. These values and the values for each individual point are listed in table 35. The advantage of examining the chi-square for each point is that it helps to identify which points in a combination survival curve agree closely with the additivity model and which values deviate from it.

chi-square	5 rad	25 rad	100 rad	250 rad	700 rad	Row Sum
0.1uM	0.614	**6.970**	1.642	**3.132**	3.743	**16.102**
1 uM	0.361	0.138	0.171	**2.318**	0.005	2.994
5 uM	0.091	0.499	0.001	**3.501**	0.348	4.441
10 uM	1.635	0.544	0.207	0.102	0.432	2.922
20 uM	0.524	**6.418**	0.016	0.873	**2.469**	**10.301**
Col Sum	3.227	**14.571**	2.038	9.927	6.998	

Table 35: Calculated chi-square values.

From table 35, the data points originating from a drug concentration of 0.1 uM form a line that is statistically different from the 100% line at a confidence level of 95%. The individual chi-square values are moderate, which suggests that the line may be genuinely different from 100% at each point. The 20 uM line is almost significantly different from the 100% line. However, it is less reasonable to believe that the line is genuinely different from 100% since only two values contribute to the large chi-square value. The same is true for the line formed by combinations with 25 rad. Only two points contribute to the statistical difference at 95%. There is evidence to suggest a pattern in the line of combinations with 250 rad, as the three lowest drug concentrations contribute to the significant chi-square (at 90%) value and imply antagonism.

The individual chi-square values were also summed over groups other than the lines associated with a given drug concentration or radiation dose. To see if there is any pattern in the data that is associated with low drug concentration and low radiation dose, the chi-square values were summed for combinations including the two lowest drug concentrations combined with the two lowest radiation doses. Notice that these combinations define a 2 by 2 group of four in the upper left-hand corner of the chi-square table above. Chi-square values for combinations of the three lowest drug concentrations and radiation doses were also summed and comprise a 3 by 3 group of nine values starting at the upper left-hand side of the above table. Similarly, the values were summed over a group of sixteen which included all values except combinations with 20 uM drug and 700 rad radiation. Finally, the chi-square values were summed over the entire treatment range. Table 36 shows the results and the critical chi-square values for rejection of the null hypothesis that the data differs from the value 100% at confidence levels of 95% and 90%.

Chi-square		Sum of 4	Sum of 9	Sum of 16	Sum of 25
		8.0858	10.490	21.933	**36.764**
95%	Alpha/2 Upper	9.348	17.535	27.488	39.364
	Alpha/2 Lower	0.216	2.180	6.262	12.401
90%	Alpha/2 Upper	7.815	15.507	24.996	36.415
	Alpha/2 Lower	0.352	2.733	7.261	13.848

Table 36: Critical chi-square values.

The group of four and of twenty-five are significant at the 90% level. The other groups are close to significance at this level of confidence. This implies a pattern tending towards a difference between additivity and combination treatments over all of the treatment range. However, the only data pairs identified as statistically different by the t-test are at the 0.1 and 20 uM points for 25 rad. The idea of a pattern for the 25 rad line has already been dismissed due to the small individual chi-square values for 1, 5 and 10 uM. Similarly for a pattern along the line of 700 rad. The pattern for 250 rad is unlikely because there are only two points for which the chi-square values are moderately large, and are consistent in the direction of deviation from 100%. According to the chi-square and t values, there is no convincing evidence for a pattern in the line of data with 0.1 uM.

Therefore, there is a difference between 100% and the survival achieved by some combination treatments. There does not seem to be any pattern that can be generalized

for the entire treatment range. Table 37 shows t-test values resulting from a comparison between each combination data point and the expected additivity value of 100%.

t-test	5 rad	25 rad	100 rad	250 rad	700 rad
0.1uM	0.783	**2.640**	1.281	1.769	-1.934
1 uM	-0.601	0.372	0.413	1.522	0.071
5 uM	-0.302	0.706	0.032	-1.871	-0.590
10 uM	1.278	0.737	-0.456	-0.320	-0.657
20 uM	0.724	**2.533**	-0.127	-0.934	-1.571

Table 37: Calculated t-test values.

Cisplatin Administration 60 Minutes After Radiation

The drug normalized combination survival curves for each drug concentration were compared to the radiation survival curve using the chi-square test. The critical chi-square value that must be equaled or exceeded in order to reject the null hypothesis that the survival curves are the same, are listed in table 38. Upper and lower critical values are given for a two-tailed test with 95% and 90% confidence levels.

chi-square	two-tailed	4 df
95%	upper alpha/2	11.143
	lower alpha/2	0.484
90%	upper alpha/2	9.488
	lower alpha/2	0.711

Table 38: Critical chi-square values.

The values depend on the number of degrees of freedom for the chi-square test which is determined by the number of points in the curves being compared. All five radiation doses, 5, 25, 100, 250 and 700 rad, were included in the determination of the chi-square values. The corresponding number of degrees of freedom for this test is 4. The values are listed in table 39.

chi-square	4 df
0.1 μM	**10.821**
1 μM	**17.332**
5 μM	2.404
10 μM	5.048
20 μM	**18.738**

Table 39: Calculated chi-square values.

The chi-square values for the curves at 0.1 uM, 1 uM and 20 uM exceed the critical chi-square values at the 90%, 95% and 95% confidence levels, respectively. The t-test values for all points on the curve should be examined to determine if the curve is genuinely different or if a single pair of data points may be influencing the result of the chi-square test.

Each combination survival curve was examined at in more detail for deviations from the radiation survival curve. Each data point on the combination survival curve was compared to the radiation survival curve at the same radiation dose using a two-sample Student's t-test. The critical values for a two-tailed test at the 95% and 90% confidence levels are listed in table 40. The number of degrees of freedom for a test comparing two samples each of size n=3, is 4. For cases in which one of the samples did not reach the end of the assay, the sample size is n=2 and the number of degrees of freedom is 2.

t-test	4 df	2 df
95%	2.776	4.303
90%	2.132	2.920

Table 40: Calculated t-test values.

For combinations involving 5, 25, 100 rad at 5 uM, only two samples were used for analysis because the third sample did not reach the end of the assay. None of these t-test values for these curves were found to be significant when compared to the radiation survival curve. The values are listed in table 41.

t-test	5 rad	25 rad	100 rad	250 rad	700 rad
0.1 uM	1.249	1.355	**2.455**	0.701	0.950
1 uM	**2.143**	1.194	**3.285**	-0.707	-0.121
5 uM	0.265	0.637	0.152	0.774	-1.141
10 uM	0.284	1.387	-0.046	-0.092	-1.741
20 uM	1.680	**2.477**	**2.930**	-0.825	-0.709

Table 41: Calculated t-test values.

For samples of size 3, five points registered as significant by the t-test. The point at 5 rad and 1 uM is significant at 90%. The points at 100 rad and 0.1, 1 and 20 uM are all significant at 95%. Finally, the point at 25 rad and 20 uM is significant at 95%. Since the chi-square values indicated that the curves along the drug concentrations of 0.1, 1 and 20 uM were significantly different from radiation alone, and not all of the t tests for pairs along these lines are significant, it is suspected the large chi-square values do not originate from a consistent difference between the combination and radiation only curves. The individual chi-square values could be examined to see if only one or two points are responsible for the significant chi-square. The values are shown in table 42.

chi-square	5 rad	25 rad	100 rad	250 rad	700 rad
0.1 uM	1.560	1.837	**6.027**	0.492	0.902
1 uM	**4.594**	1.426	**10.796**	0.501	0.014
5 uM	0.070	0.406	0.023	0.600	1.302
10 uM	0.081	1.924	0.002	0.008	3.032
20 uM	2.824	**6.137**	**8.589**	0.682	0.503

Table 42: Calculated chi-square values.

Since the chi-square value is just the square of the value returned by the t-test, the t values could be used to obtain the desired information. However, the individual chi-square values can more easily be compared to the sum returned by the chi-square test. It is obvious that the value at 100 rad and 0.1 uM that was found to be significant by the t-test is very influential in causing the chi-square test to return a significant value. Subtracting off this point from the chi-square test and comparing the value to the critical value at 3 degrees of freedom, results in a chi-square test that is no longer significant. Likewise for the 1 uM curve when the point at 100 rad is removed form the chi-square analysis. However, the point at 5 rad is also significant by the t-test for the 1 uM curve and it contributes moderately to the chi-square. There is, therefore, some evidence to suggest that this curve is different from the radiation alone curve and this evidence is more convincing than that for a difference between radiation alone and the 0.1 uM curve. The individual chi-square values for the three lowest radiation doses are all substantial. The points at 25 and 100 rad are statistically different from radiation only as determined by the t-test. The consistency in large individual chi-square values strongly indicates a curve that is different from radiation alone, especially for the lower radiation doses.

The t values retain information about the direction of deviation of combination data points from the radiation line, whereas chi-square does not because it squares the deviations. The t values show that for the significant points, the deviation from the radiation only curve is in the direction of increased survival. In general, over the radiation dose range 5 to 100 rad, the deviations are in this direction. This suggests a pattern in the direction of increased survival relative to radiation alone for radiation doses that are not large.

The radiation normalized combination survival curves for each radiation dose were compared to the drug survival curve using the chi-square test. The critical chi-square values that must be equaled or exceeded in order to reject the null hypothesis that the survival curves are the same, are the same as previously used. Upper and lower critical values are given for a two-tailed test with 95% and 90% confidence levels in table 43.

chi-square	4 df
5 rad	7.496
25 rad	**47.339**
100 rad	**13.489**
250 rad	3.1093
700 rad	**24.509**

Table 43: Calculated chi-square values.

The chi-square tests show that the curve for 25 rad is statistically different from the drug survival curve at the 95% level. This suggest that for 25 rad, the majority of the combination points are different from drug alone. This suggestion will be examined in

more detail by looking at the individual chi-square and t-test values. The curve for 5 rad has a moderately large, although not significant, value for the chi-square tests. It is not known whether this is due to a consistent difference between the combination curve and drug alone or if a single data pair greatly influences the size of the chi-square value returned. The chi-square values for the 100 and 700 rad curves are significant at the 95% level. Again, the individual chi-square and t values should be examined to determine the consistency of fit between the drug only and the combination curves over all data pairs. The values are displayed in table 44.

chi-square	0.1 uM	1 uM	5 uM	10 uM	20 uM
5 rad	**3.515**	**2.334**	0.001	0.018	1.626
25 rad	**2.647**	**4.400**	**39.197**	0.738	0.361
100 rad	**6.230**	**6.742**	0.028	0.0006	0.486
250 rad	**2.453**	0.188	0.376	0.083	0.007
700 rad	**2.055**	0.263	**15.673**	**5.515**	1.001

Table 44: Calculated chi-square values.

The individual chi-square values show the moderately large chi-square test value for the 5 rad curve originates from both the 0.1 and 1 uM points, although neither of these points is significant by the t-test. The chi-square test for the curve at 25 rad is very much influenced by the point at 5 uM which is significant for a t-test at 95% confidence level. However, the points at 0.1 and 1 uM are substantial and the point at 1 uM is almost significant at the 90% level. It appears that a pattern exists where for the low drug concentrations, the combination curves deviate from the drug only curve. The curve at 100 rad is consistent with this statement. The individual chi-square values from the two lowest drug concentrations both contribute to the chi-square test value. The chi-square

test value for the curve with 700 rad is largely influenced by the value at 5 uM, which is significant at 95%. The point at 10 uM is also significant, at 90%, and contributes to the chi-square test value. Even the individual chi-square value for the point at 0.1 uM is not trivial. This implies that combinations with 700 rad deviate from he drug only line for all drug concentrations. The t-test values are listed in table 45.

t-test	0.1 uM	1 uM	5 uM	10 uM	20 uM
5 rad	1.874	1.527	0.033	0.137	1.275
25 rad	1.627	2.097	**6.260**	0.859	0.600
100 rad	**2.496**	**2.596**	0.169	-0.025	0.697
250 rad	1.566	-0.434	0.613	0.289	-0.085
700 rad	1.433	-0.513	**-3.958**	**-2.348**	-1.000

Table 45: Calculated t-test values.

It can be seen from the table of t values that the direction of deviation from the drug only curve for the curves with 5, 25 and 100 rad is, in general, in the direction of increased survival. This pattern is not seen in the curve for 250 rad, where the direction of deviation is not consistent for increasing drug concentration. The majority of points on the 700 rad curve deviate in the direction of decreased survival as indicated by the t values of opposite sign. Therefore, a pattern is present which implies that combination treatment results in less cell kill than drug alone, especially for relatively low drug concentrations and radiation doses.

In order to compare combination treatments over the entire radiation dose range and drug concentration range, the survival data was normalized to both radiation and drug

data. The corrected combination data points were then compared for a given radiation dose and also for a given drug concentration to identify any patterns in combination response. Corrected data points were first grouped by drug concentration and compared using an analysis of variance. The F values and corresponding probabilities are listed in table 46.

F-test	0.1 uM	1 uM	5 uM	10 uM	20 uM
F	0.218	0.722	1.665	0.764	0.639
P(F)	0.921	0.595	0.260	0.571	0.646

Table 46: Calculated F-test values.

None of the F values were significant. This means that the combination response was the same for a given drug concentration despite the radiation dose used.

The corrected data points were also grouped according to radiation dose. As can be seen from the F values shown in table 47, there are no points of statistical significance. This means that at least no one combination performs better with respect to cell kill than any other combination for a given radiation dose.

F-test	5 rad	25 rad	100 rad	250 rad	700 rad
F	1.184	0.644	0.836	0.426	0.630
P(F)	0.386	0.646	0.538	0.820	0.681

Table 47: Calculated F-test values.

It is difficult to discuss the relative roles of radiation and drug in producing cell death in combination, as almost all of the combination treatments produced the same surviving fraction within error.

The combination data corrected for drug and radiation were compared to the value predicted by the additivity model of 100%. The chi-square test was performed comparing the combination survival curves to a straight line through 100% survival. The data was first grouped and compared according to drug concentration then according to radiation dose. These values and the values for each individual point are listed in table 48. The advantage of examining the chi-square for each point is that it helps to identify which points in a combination survival curve agree closely with the additivity model and which values deviate from it.

Chi-square	5 rad	25 rad	100 rad	250 rad	700 rad	Row Sum
0.1uM	**2.919**	**2.419**	**9.164**	0.836	2.507	**17.846**
1 uM	**2.964**	**2.692**	**7.621**	0.064	0.161	**13.503**
5 uM	0.0001	**1381.792**	0.034	0.725	**5.196**	**1387.749**
10 uM	0.071	1.140	0.001	0.077	**13.171**	**14.461**
20 uM	**2.568**	**2.629**	**6.046**	0.214	0.006	**11.466**
Col Sum	8.524	**1390.673**	**22.868**	1.918	**21.043**	

Table 48: Calculated chi-square values.

Table 48 shows that the curves including 0.1, 1, 5, 10, or 20 uM are all significantly different from the 100% line at the 95% level. The difference is consistent across the entire radiation dose range for 0.1 uM and across the 5 to 100 rad dose range for 1 uM. At 5 uM the data is less convincing that a pattern exist across the whole range

of radiation doses, however, there are two points which are clearly different. The point at 700 rad seems to be the only point on the 10 uM line that is different from 100%. The three lowest radiation doses at 20 uM also suggest that a pattern exist for differences over the range of low radiation doses. The curves for a given radiation dose are significant at the 95% level for 25, 100 and 700 rad. The 5 rad curve is almost significant at the 90% level. For 5 rad, the two lowest drug concentrations seem to be clearly different, as does the point at 20 uM.. Most of the data points along the 25 rad curve show a contribute to the statistical difference, which implies that the 25 rad combination line is consistently different from 100%. The 100 and 700 rad curves are also comprised of several points that contribute to a significant difference.

The direction of the differences pointed out in the paragraph above are important in describing the ability of combinations to achieve cell kill, relative to the additivity model. Hence, the direction implies a synergistic interaction if the deviation is in the negative direction and an antagonistic interaction if the deviations are positive.

t-test	5 rad	25 rad	100 rad	250 rad	700 rad
0.1uM	1.708	1.555	**3.027**	0.914	1.583
1 uM	1.721	1.640	**2.760**	-0.254	-0.401
5 uM	0.024	**37.172**	0.185	0.851	**-2.279**
10 uM	0.265	1.068	-0.038	0.278	**-3.629**
20 uM	1.602	1.621	**2.459**	-0.463	-0.082

Table 49: Calculated t-test values.

The curves with 0.1, 1 and 20 uM show a consistent positive deviation from 100% over the range of radiation doses 5 to 100 rad. The 5 uM curve was thought to be

consistent with this pattern, however, it can be seen from the t values that only the point at 25 rad deviates in the positive direction and the other point originally thought to have provided evidence for a pattern of difference deviates in the other direction. The 10 uM is not consistently positive or negative. In general, for relatively low radiation doses and drug concentrations, combination treatment results in an antagonistic interaction. This is also supported by a consistent and substantial positive deviation over the drug concentration range 0.1 and 1 uM for curves with 5, 25 and 100 rad. A pattern toward synergistic interaction seems to exist for 700 rad at the higher drug concentrations.

The individual chi-square values were also summed over groups other than the lines associated with a given drug concentration or radiation dose. To see if there is any pattern in the data that is associated with low drug concentration and low radiation dose, the chi-square values were summed for the two lowest drug concentrations combined with the two lowest radiation doses. Notice that these combinations define a group of four (a 2 by 2 square) in the upper left-hand corner of the chi-square table above. Chi-square values were also summed for the three lowest drug concentrations and three lowest radiation doses. This groups forms a 3 by 3 square of nine starting at the upper left-hand side of the above table. Similarly, the values were summed over a group of sixteen which included all values except combinations with 20 uM drug and 700 rad radiation. Finally, the chi-square values were summed over the entire treatment range of 25 values. Table 50 shows the results and the critical chi-square values for rejection of the null hypothesis that the data differs from the value 100% at confidence levels of 95% and 90%.

chi-square		Sum of 4	Sum of 9	Sum of 16	Sum of 25
		10.995	1409.608	1412.525	1445.028
95%	alpha/2 upper	9.348	17.535	27.488	39.364
	alpha/2 lower	0.216	2.180	6.262	12.401
90%	alpha/2 upper	7.815	15.507	24.996	36.415
	alpha/2 lower	0.352	2.733	7.261	13.848

Table 50: Critical chi-square test values.

Each group is significant at the 95% level of confidence, even when the point at 5 uM and 25 rad which has an individual chi-square value of 1381.792 is not included. This implies a pattern tending towards a difference between additivity and combination treatments over all of the treatment range. This pattern is supported by the individual chi-square and t-test values. Therefore, there is a difference between 100% and the survival achieved by some combination treatments, and the difference is consistently in the direction of antagonism for low radiation doses and drug concentrations.

Statistical Analysis for SCC-25/CP Cells

Radiation Administration 60 Minutes Before Cisplatin

The SCC-25 cells were treated simultaneously with the SCC-25/CP cells to ensure that differences observed between the results for the two cell lines are not due to the method. The SCC-25 combination data corrected for both drug and radiation were compared with the additivity line of 100% survival. No points were found to be statistically different from additivity. However, the points in the 10 uM line for all radiation doses consistently vary from 100% in the direction of antagonism. The t-test values are in table 51.

t-test	5 rad	25 rad	100 rad	250 rad	700 rad
0.1 uM	-1.146	-0.251	0.975	1.153	1.429
10 uM	**1.675**	**1.341**	**1.117**	**1.707**	**1.5057**

Table 51: Calculated t-test values for SCC-25 cells.

The drug normalized combination data for the SCC-25/CP cell line were compared to the radiation only line by the chi-square test. The curve at 50 uM was found to be statistically significant at the 95% level of confidence. The values for 50 and 10 uM are listed in table 52.

chi-square	4 df
10 uM	5.864804
50 uM	**23.99428**

Table 52: Calculated chi-square values for SCC-25/CP cells.

The individual chi-square values in table 53 show that all of the data points along the 50 uM line contribute to the chi-square test value. This implies that the curve at 50 uM is genuinely different from the radiation only curve.

chi-square	5 rad	25 rad	100 rad	250 rad	700 rad
10 uM	3.701	0.275	1.718	0.021	0.148
50 uM	**3.014**	**3.942**	**12.290**	**2.596**	**2.149**

Table 53: Calculated chi-square values for SCC-25/CP cells.

The t-test values show that only one point in the two curves is statistically significant. The t values are shown in table 54.

t-test	5 rad	25 rad	100 rad	250 rad	700 rad
10 uM	-1.923	-0.524	1.310	0.146	-0.384
50 uM	-1.736	-1.985	**-3.505**	-1.611	-1.466

Table 54: Calculated t-test values for SCC-25/CP cells.

According to the chi-square values, the radiation corrected data deviate from the drug only curve only for 50 uM at 700 rad. These points are not significant at the levels tested using the t-test. The t-test values are listed in table 55. The chi-square and t-test values are listed in table 56.

t-test	5 rad	25 rad	100 rad	250 rad	700 rad
10 uM	1.842	1.085	-1.187	-.685	1.039
50 uM	0.823	1.271	1.024	2.001	**2.502**

Table 55: Calculated t-test values for SCC-25/CP cells.

chi-square	5 rad	25 rad	100 rad	250 rad	700 rad
10 uM	3.392	1.177	1.410	.469	1.081
50 uM	0.677	1.616	1.048	4.007	**6.261**

Table 56: Calculated chi-square values for SCC-25/CP cells.

The data normalized to both drug and radiation are significantly different from the additivity line of 100% across the entire radiation dose range for 50 uM. The individual chi-square values are consistent with the significance determine by the t-test. The corresponding chi-square test for the 50 uM line is very large. According to the t values, the deviation from additivity is in the direction of decreased survival. This provides strong evidence that the combinations including 50 uM of drug are synergistic for all radiation doses examined. The t-test values are shown in table 57. The chi-square values are listed in table 58.

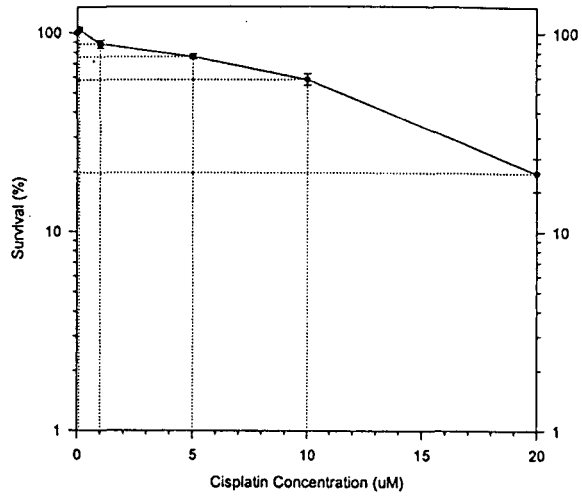
t-test	5 rad	25 rad	100 rad	250 rad	700 rad
10 uM	-1.871	-0.843	1.023	1.026	-1.141
50 uM	-1.352	**-3.830**	**-12.291**	**-2.744**	**-3.946**

Table 57: Calculated t-test values for SCC-25/CP cells.

chi-square	5 rad	25 rad	100 rad	250 rad	700 rad	Sum chi
10 uM	3.503	0.711	1.047	1.053	1.302	6.317
50 uM	**1.828**	**14.671**	**151.082**	**7.530**	**15.573**	**175.113**

Table 58: Calculated chi-square values for SCC-25/CP cells.

Not enough drug concentration were explored for each radiation dose to be able to tell if a pattern of exists for a given radiation dose over the drug concentration range.



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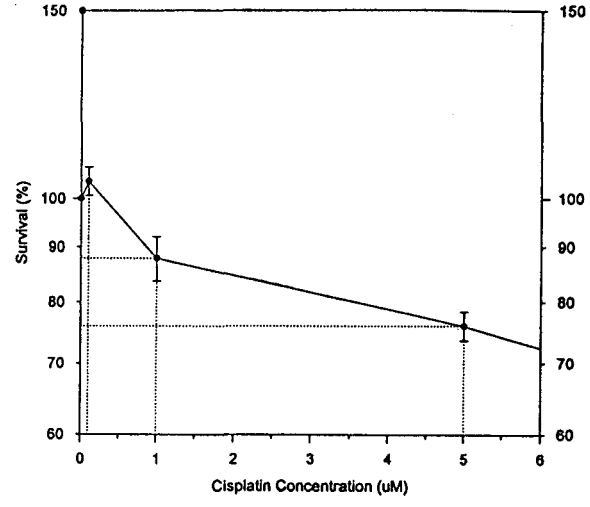
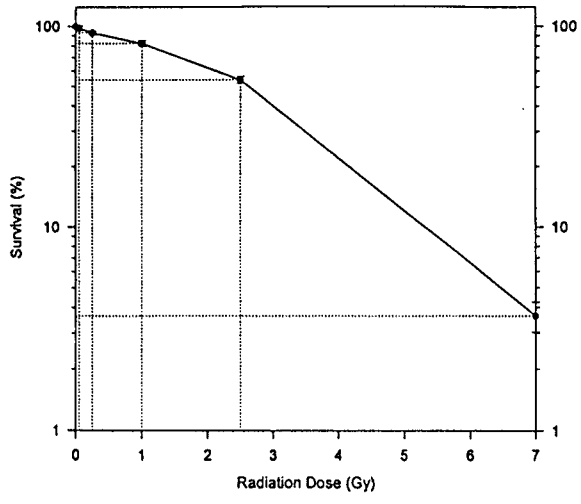


b)

Figure 1: Cisplatin survival curve for SCC-25 cells plotted for concentrations ranging from a) 0.1 to 20 uM and b) from 0.1 to 5 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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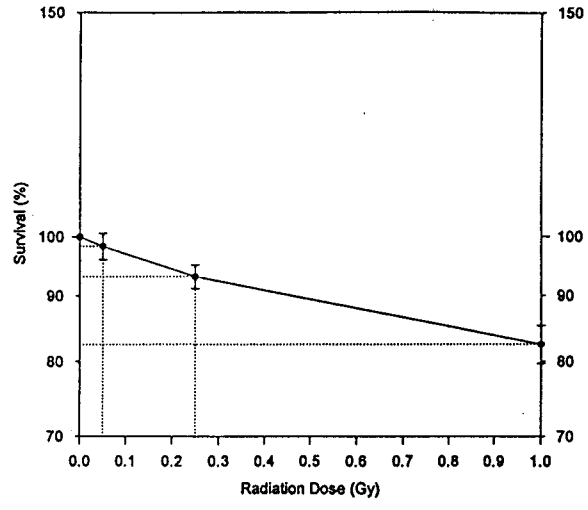


b)

Figure 2: Radiation survival curve for SCC-25 cells plotted for doses ranging from a) 0.05 to 7.0 Gy and b) from 0.05 to 1.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*

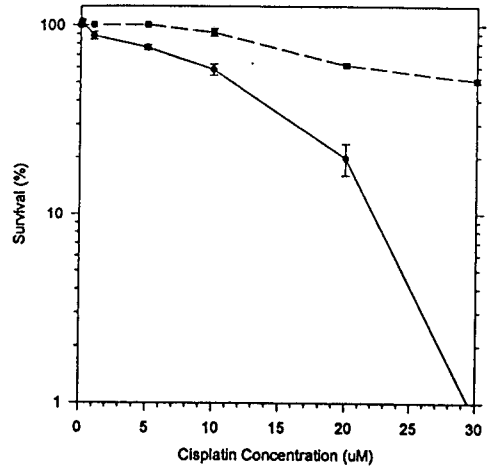
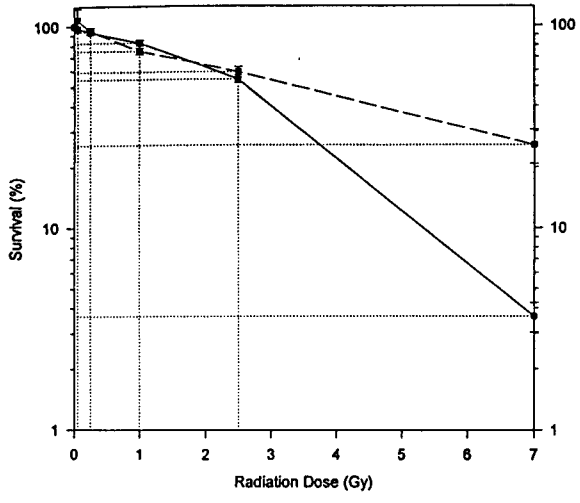


Figure 3: Cisplatin survival curve for SCC-25 cells (solid line) and for SCC-25/CP cells (dashed line) plotted for concentrations ranging from 0.1 to 30 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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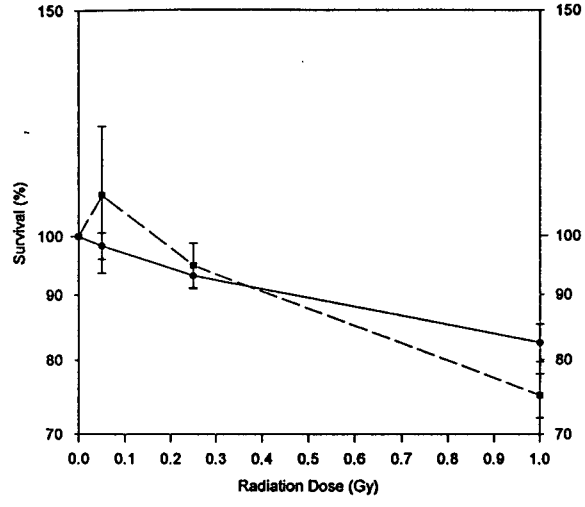
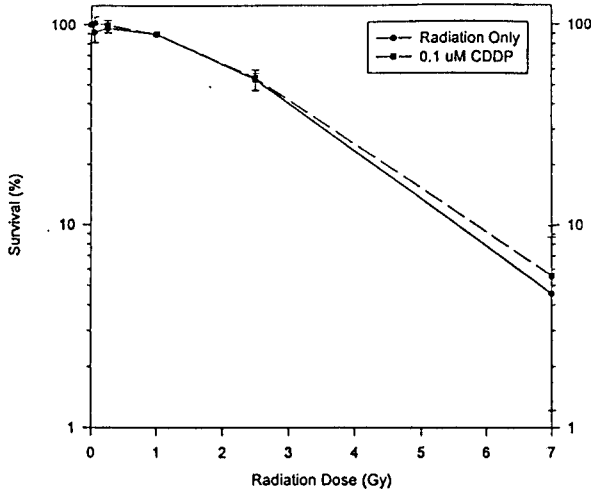


b)

Figure 4: Radiation survival curve for SCC-25 cells (solid line) and for SCC-25/CP cells (dashed line) plotted for doses ranging from a) 0.05 to 7.0 Gy and b) from 0.05 to 1.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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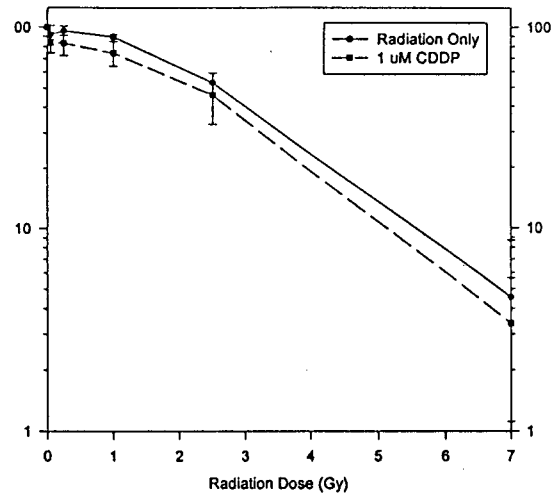

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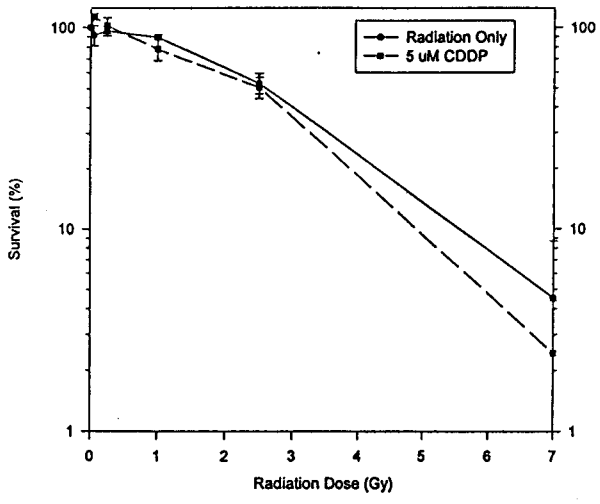

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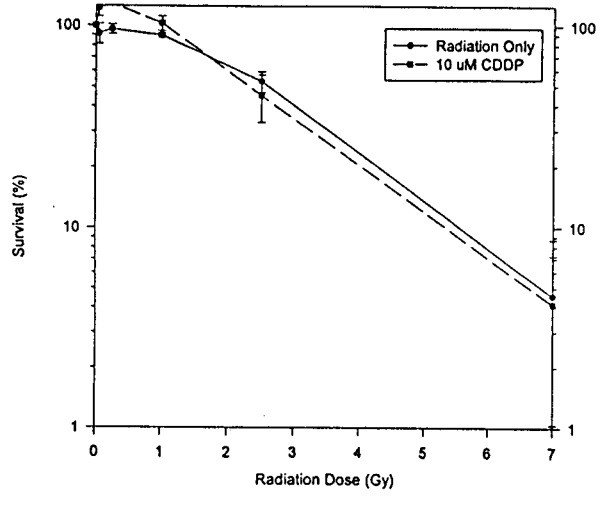

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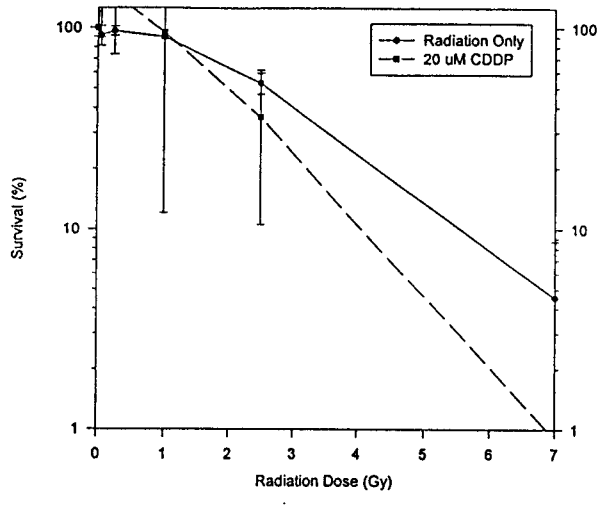
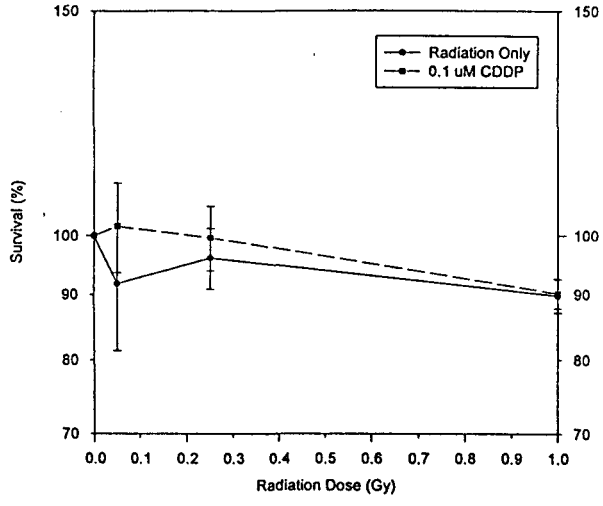


e)

Figure 5: Radiation survival curves for SCC-25 cells treated with cisplatin 60 minutes before radiation. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin pretreatment are shown in relation to the radiation survival curve without cisplatin pretreatment. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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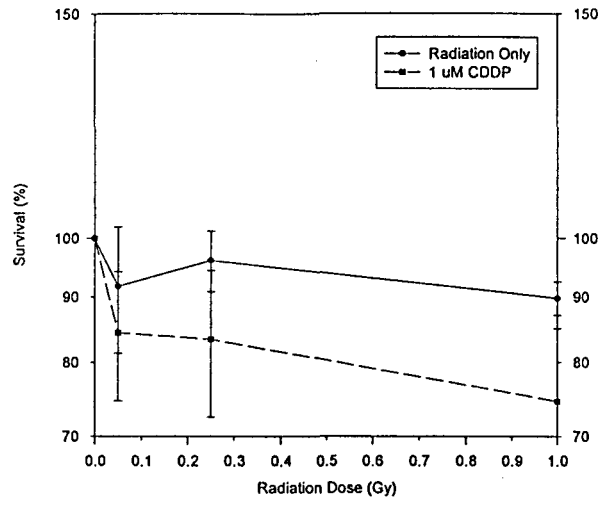

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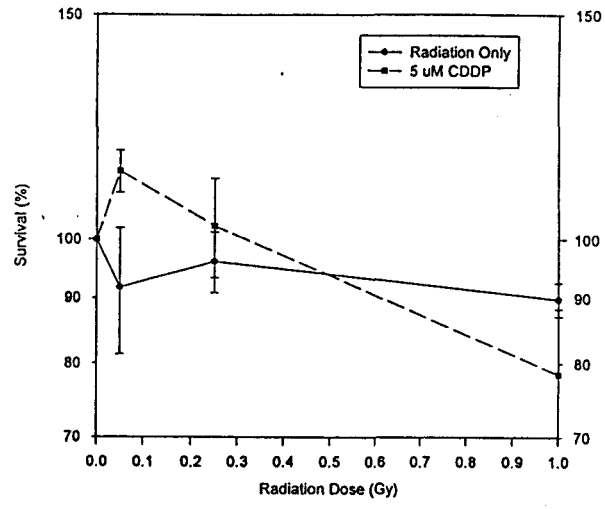

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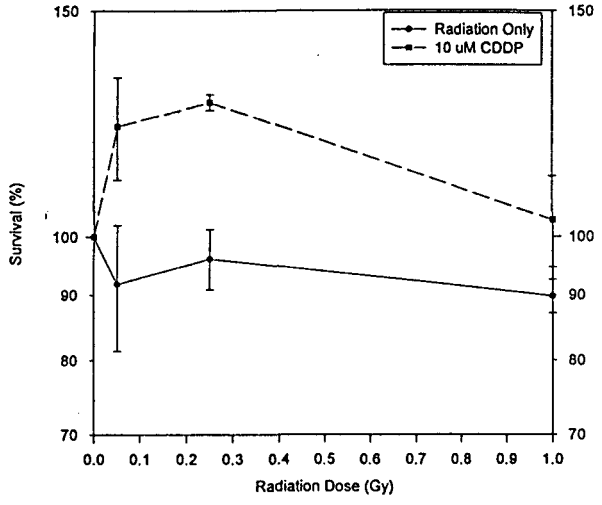

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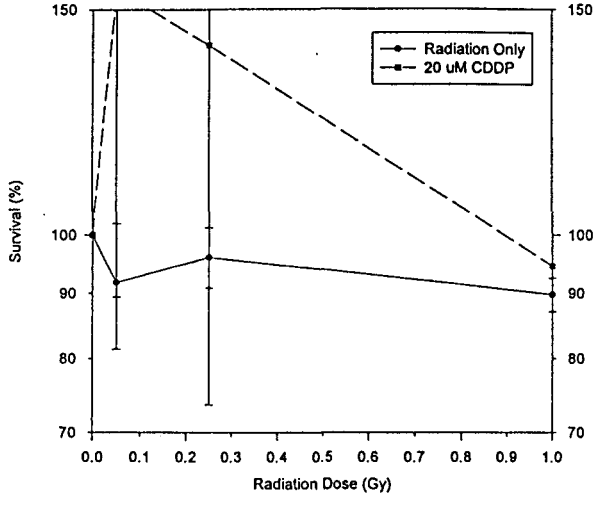
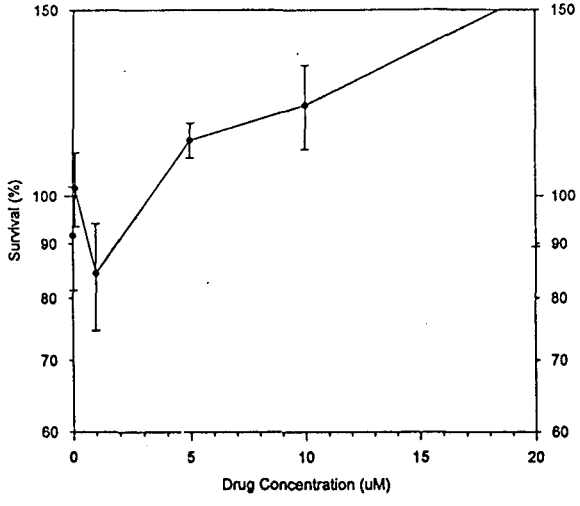


e)

Figure 6: Radiation survival curves for SCC-25 cells treated with cisplatin 60 minutes before radiation. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin pretreatment are shown in relation to the radiation survival curve without cisplatin pretreatment. The curves are plotted for radiation doses ranging from 0.05 to 1.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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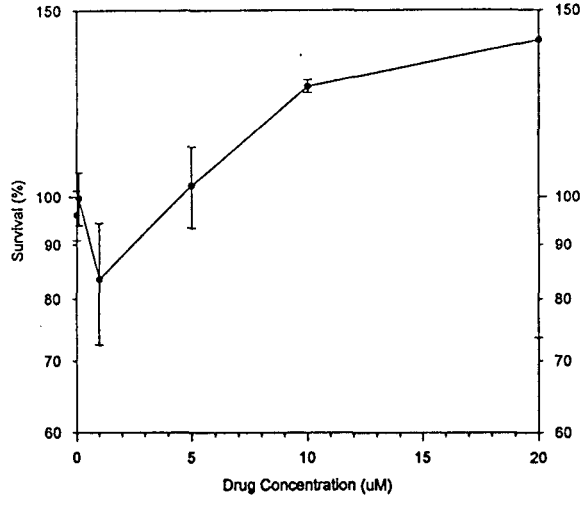

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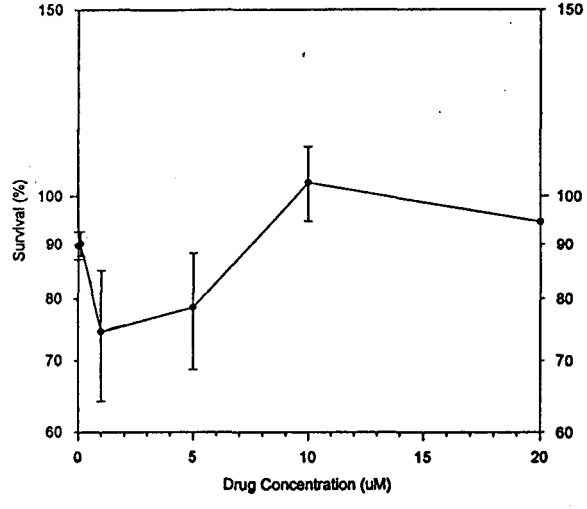

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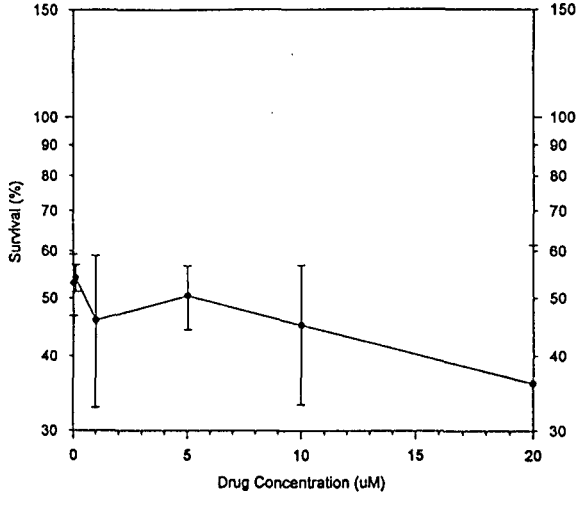


d)

Figure 7: Radiation survival for SCC-25 cells plotted as a function of cisplatin pretreatment concentration received 60 minutes before radiation. The curves for a) 0.05 Gy, b) 0.25 Gy, c) 1.0 Gy and d) 2.5 Gy radiation are shown. The curves are plotted for cisplatin pretreatment concentrations ranging from 0.1 to 20 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*

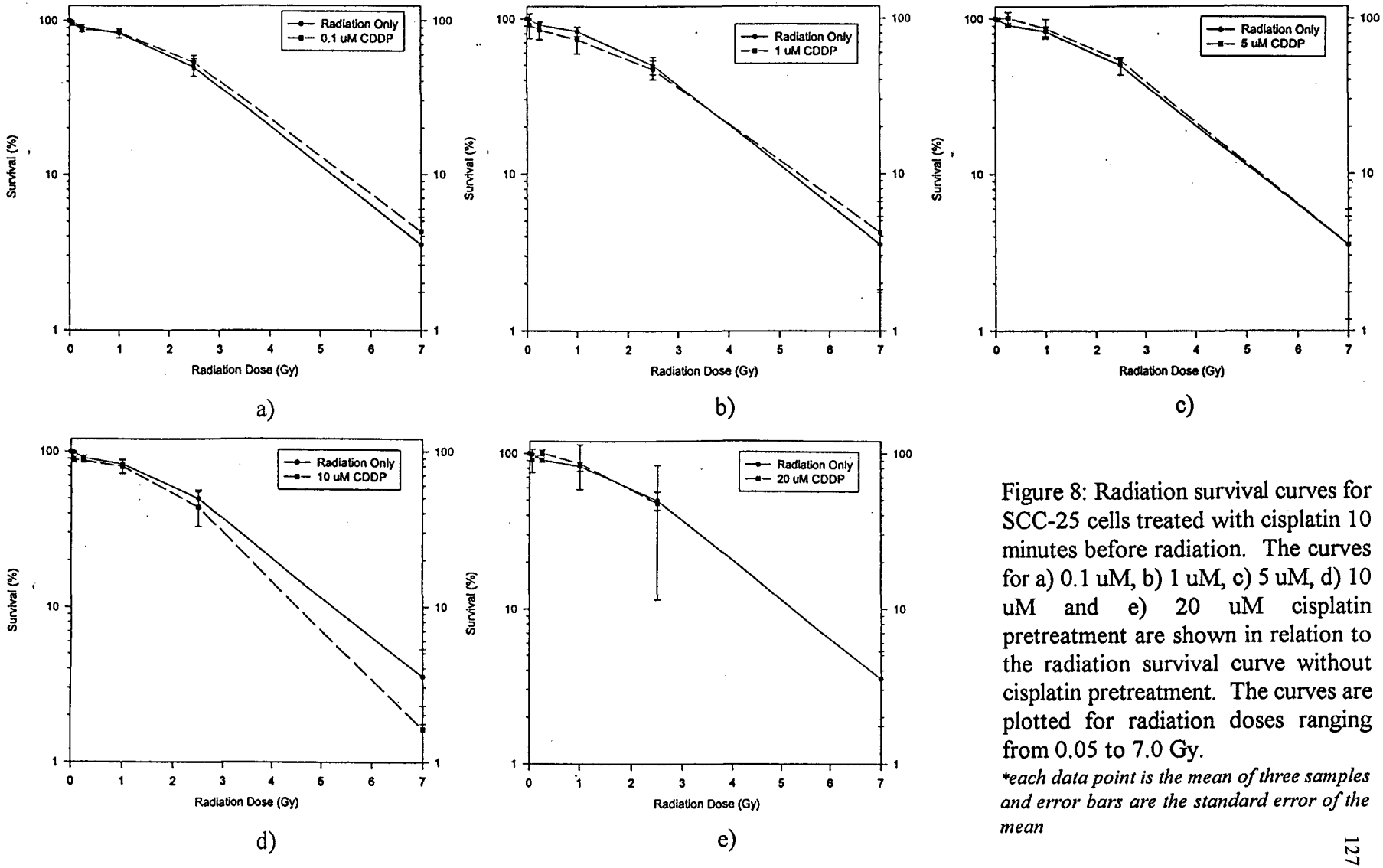


Figure 8: Radiation survival curves for SCC-25 cells treated with cisplatin 10 minutes before radiation. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin pretreatment are shown in relation to the radiation survival curve without cisplatin pretreatment. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*

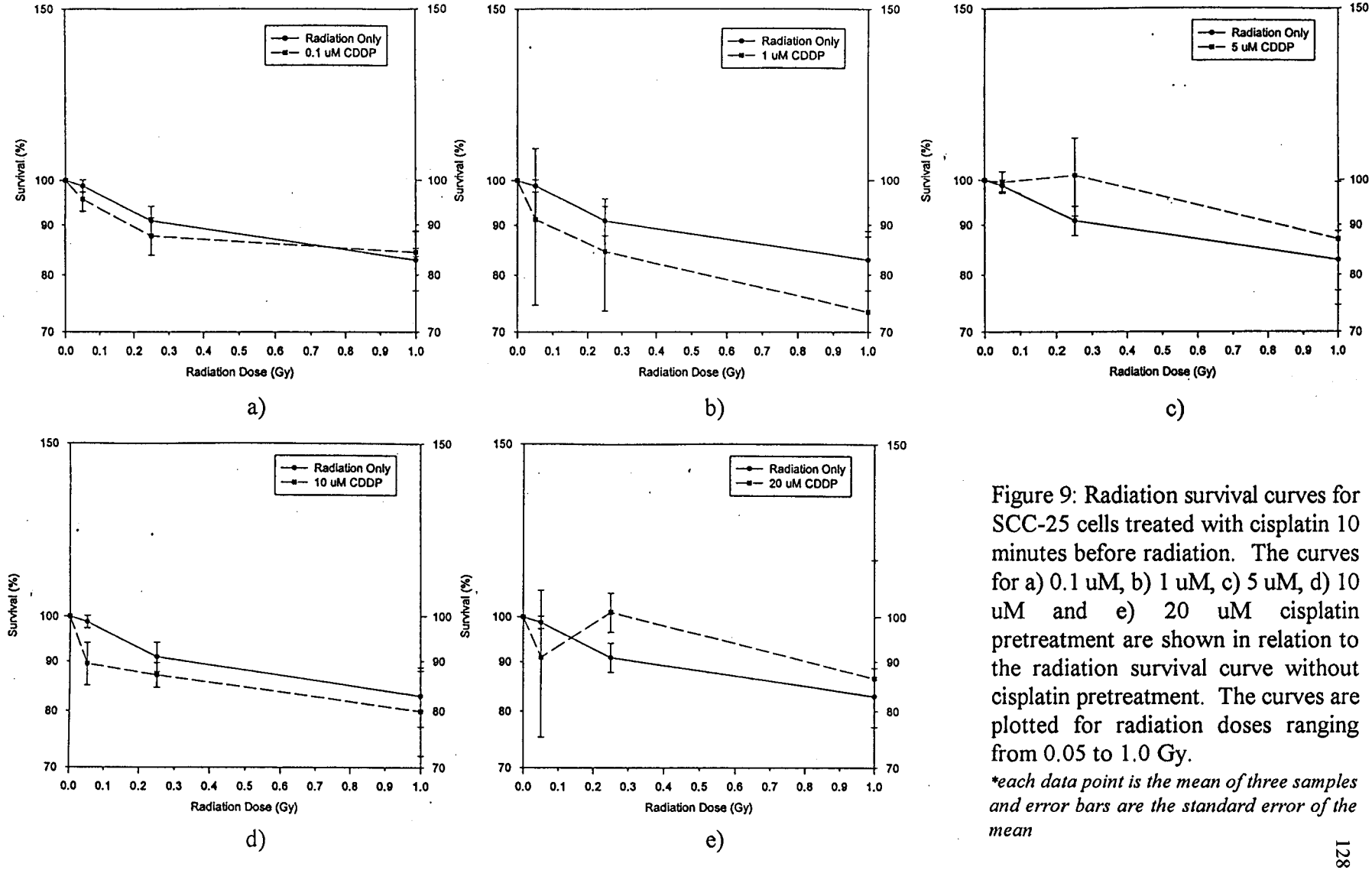


Figure 9: Radiation survival curves for SCC-25 cells treated with cisplatin 10 minutes before radiation. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin pretreatment are shown in relation to the radiation survival curve without cisplatin pretreatment. The curves are plotted for radiation doses ranging from 0.05 to 1.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*

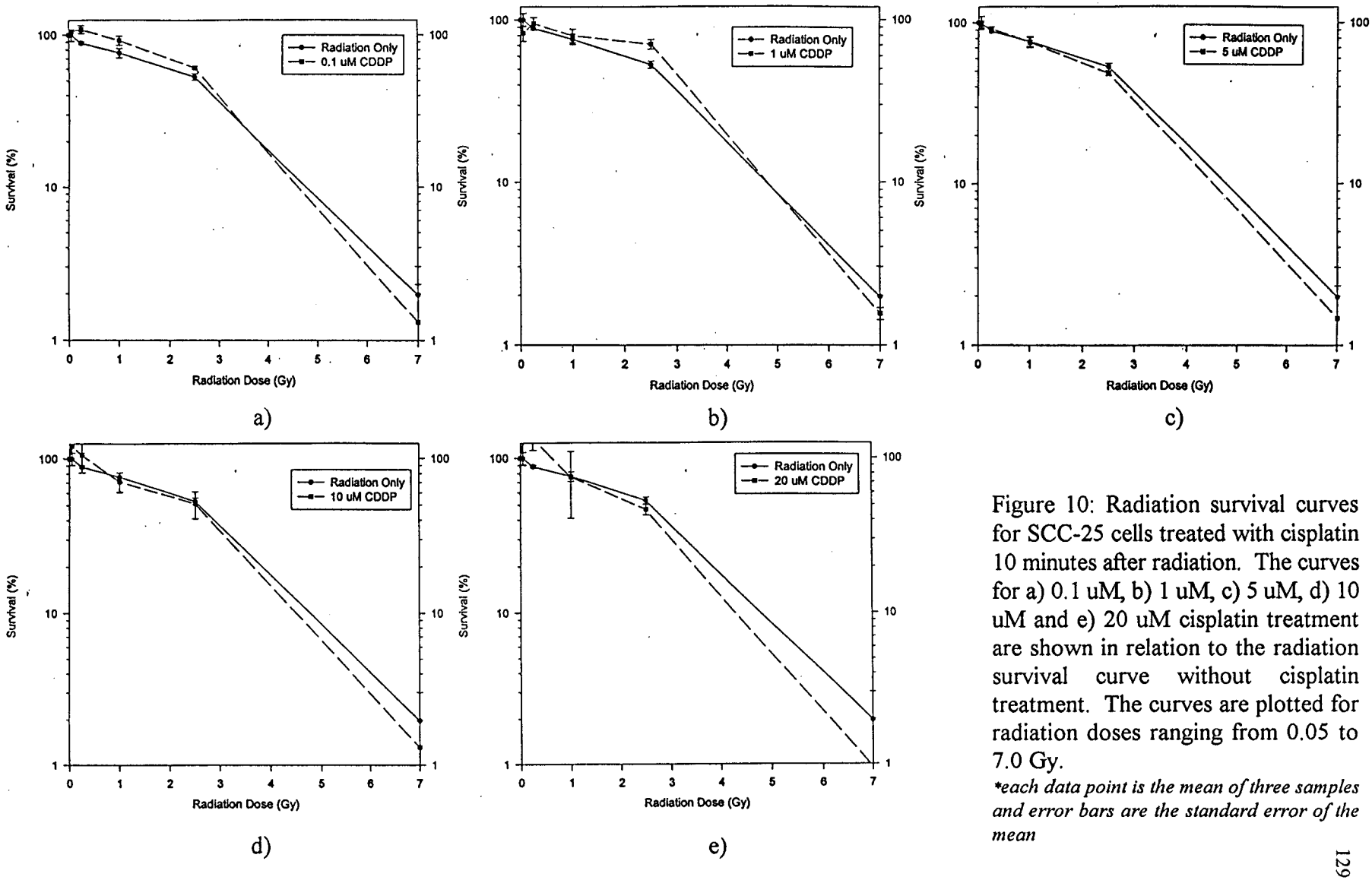


Figure 10: Radiation survival curves for SCC-25 cells treated with cisplatin 10 minutes after radiation. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin treatment are shown in relation to the radiation survival curve without cisplatin treatment. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*

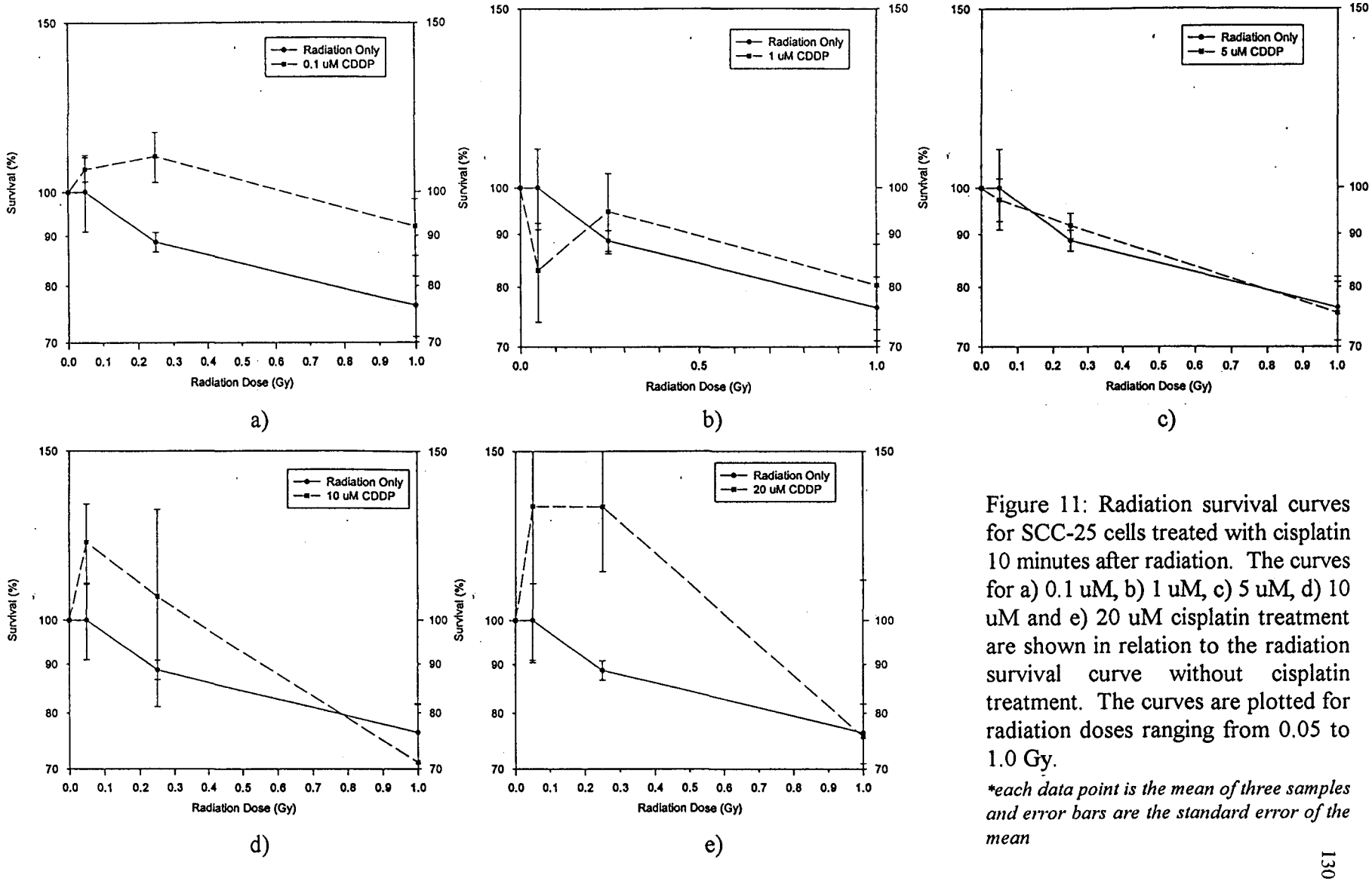
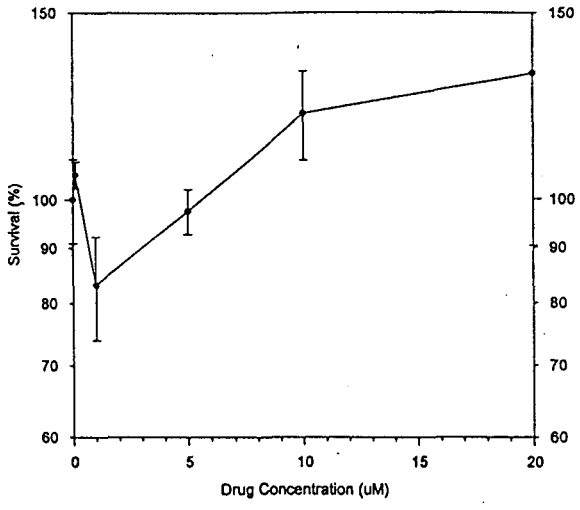


Figure 11: Radiation survival curves for SCC-25 cells treated with cisplatin 10 minutes after radiation. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin treatment are shown in relation to the radiation survival curve without cisplatin treatment. The curves are plotted for radiation doses ranging from 0.05 to 1.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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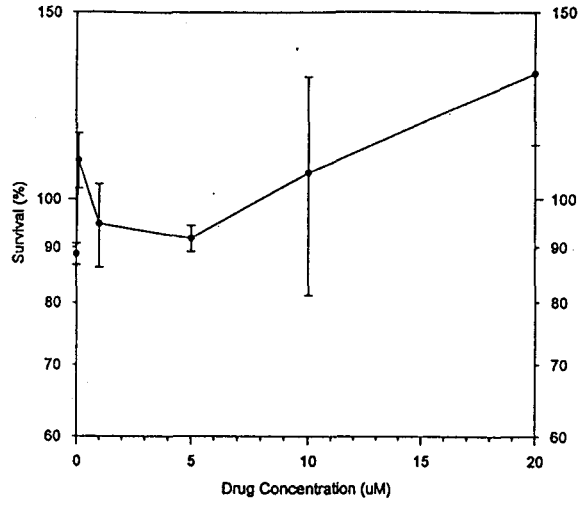

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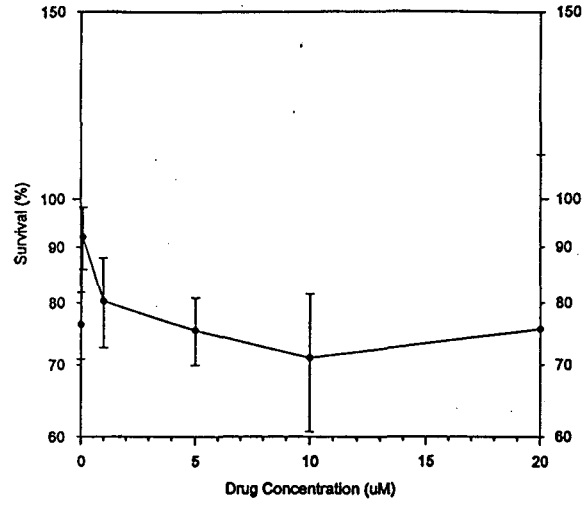

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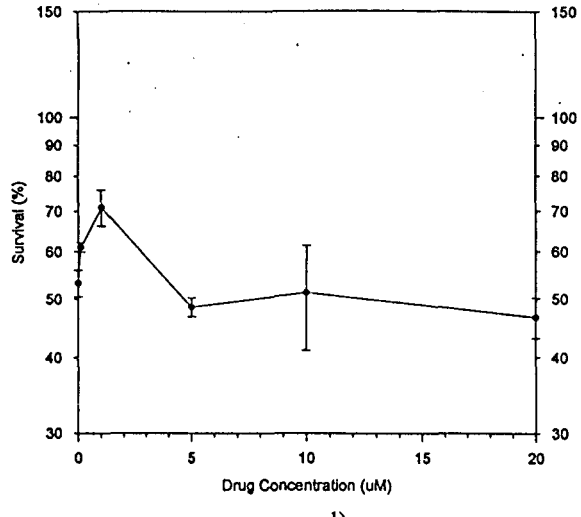


d)

Figure 12: Radiation survival for SCC-25 cells plotted as a function of cisplatin concentration received 10 minutes after radiation. The curves for a) 0.05 Gy, b) 0.25 Gy, c) 1.0 Gy and d) 2.5 Gy radiation are shown. The curves are plotted for cisplatin concentrations ranging from 0.1 to 20 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*

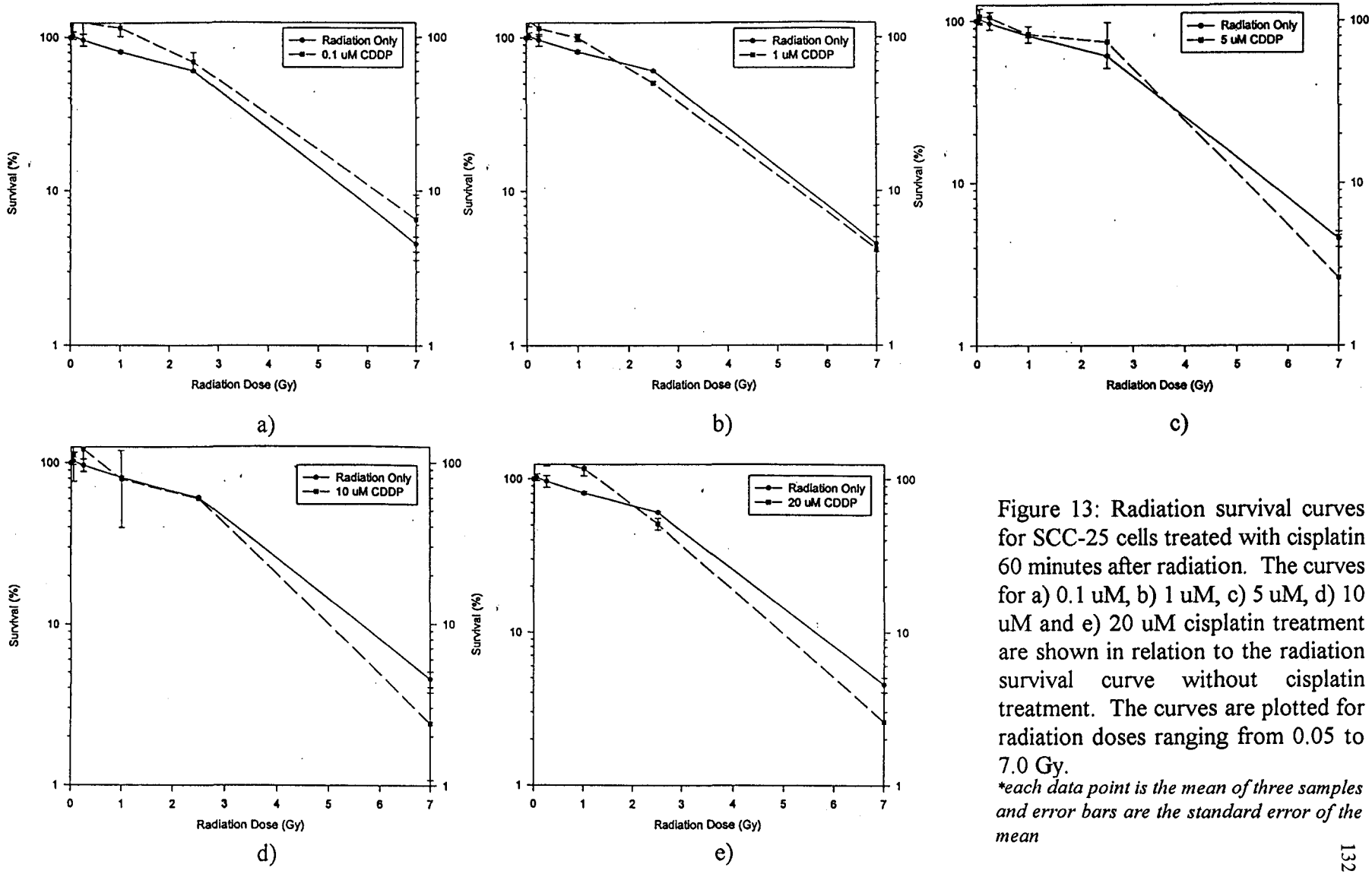


Figure 13: Radiation survival curves for SCC-25 cells treated with cisplatin 60 minutes after radiation. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin treatment are shown in relation to the radiation survival curve without cisplatin treatment. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*

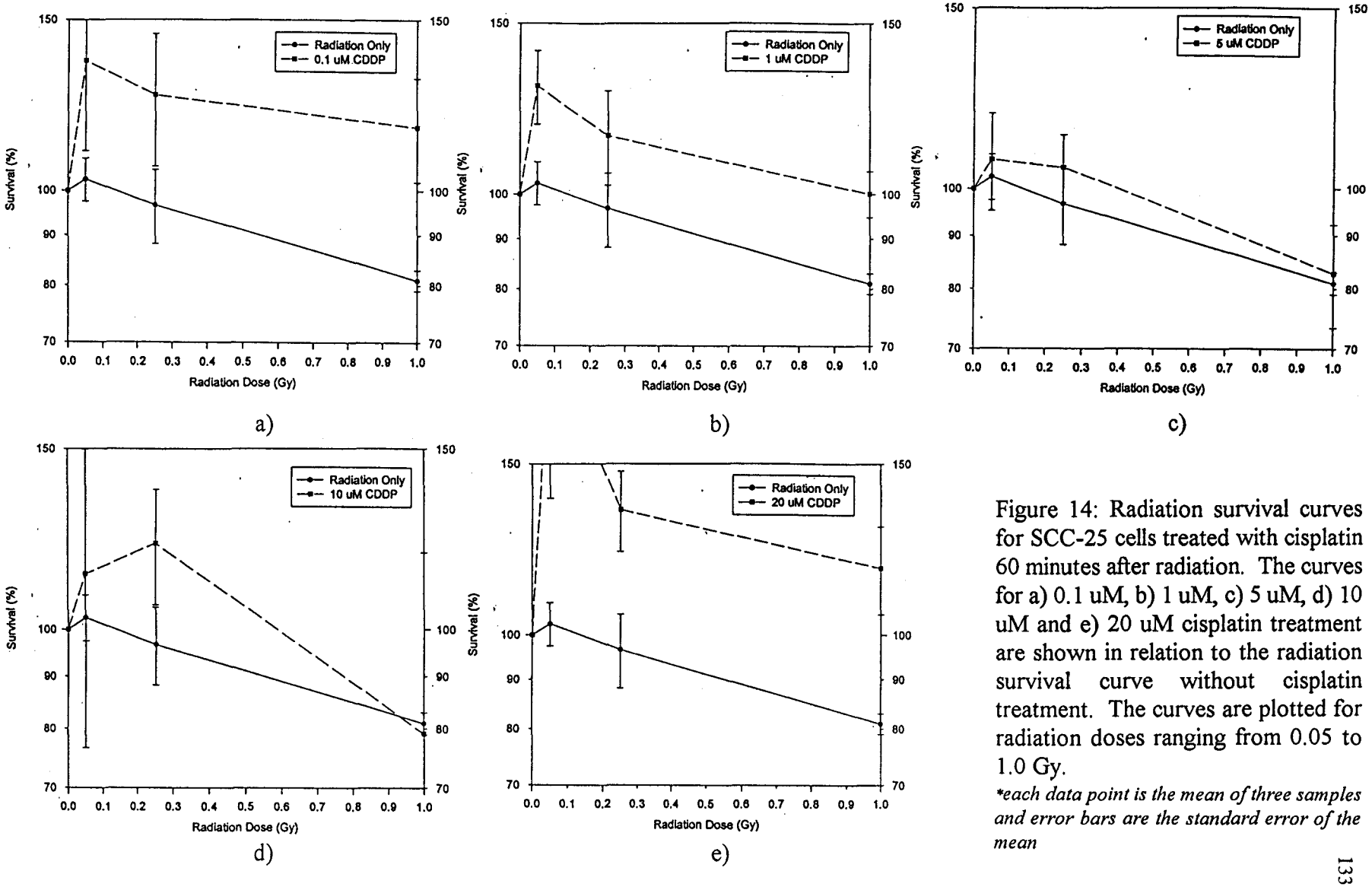
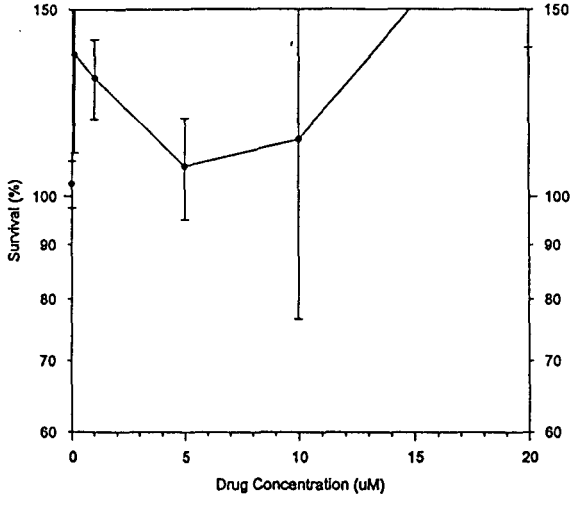


Figure 14: Radiation survival curves for SCC-25 cells treated with cisplatin 60 minutes after radiation. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin treatment are shown in relation to the radiation survival curve without cisplatin treatment. The curves are plotted for radiation doses ranging from 0.05 to 1.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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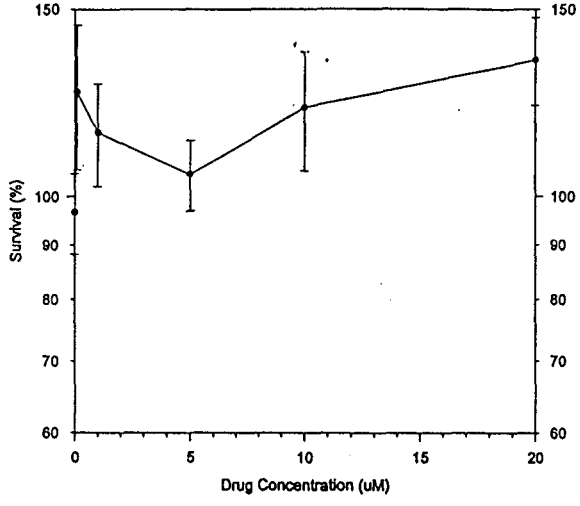

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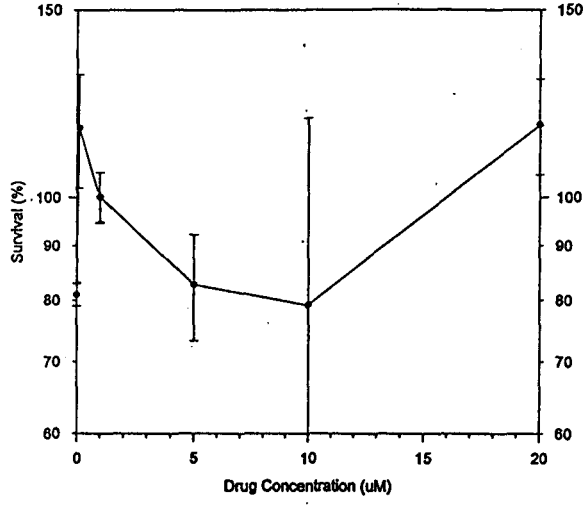

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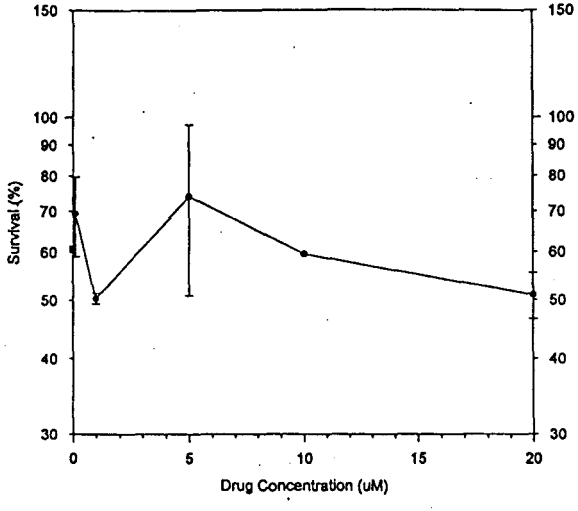
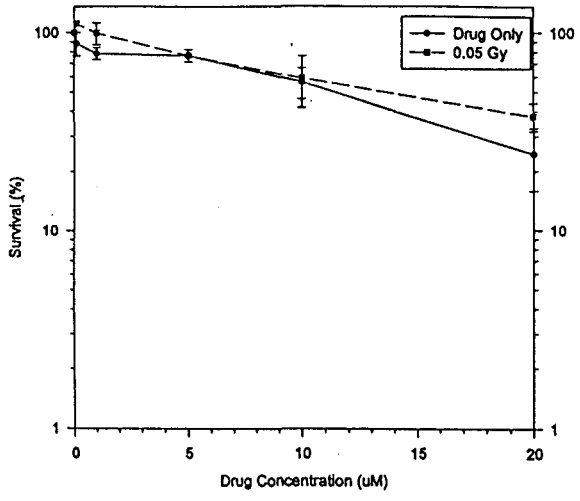


d)

Figure 15: Radiation survival for SCC-25 cells plotted as a function of cisplatin concentration received 60 minutes after radiation. The curves for a) 0.05 Gy, b) 0.25 Gy, c) 1.0 Gy and d) 2.5 Gy radiation are shown. The curves are plotted for cisplatin concentrations ranging from 0.1 to 20 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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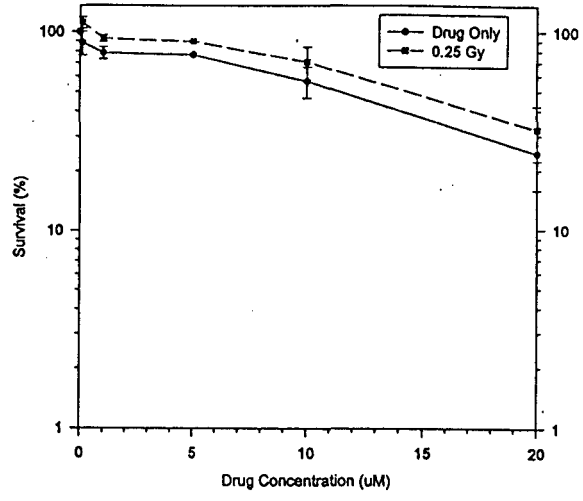

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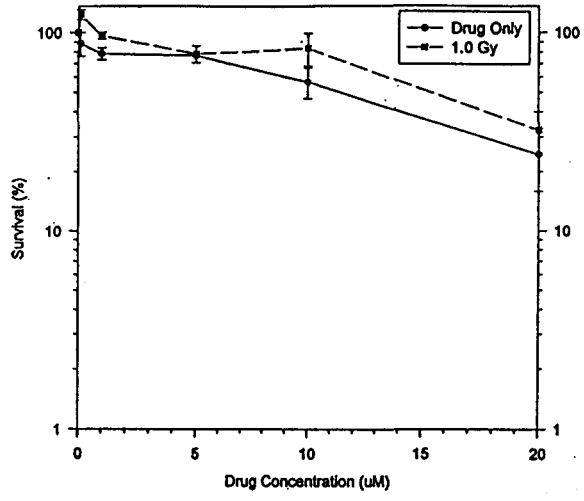

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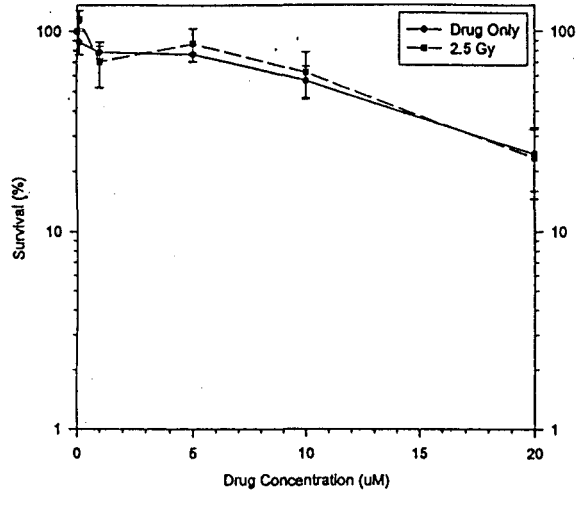

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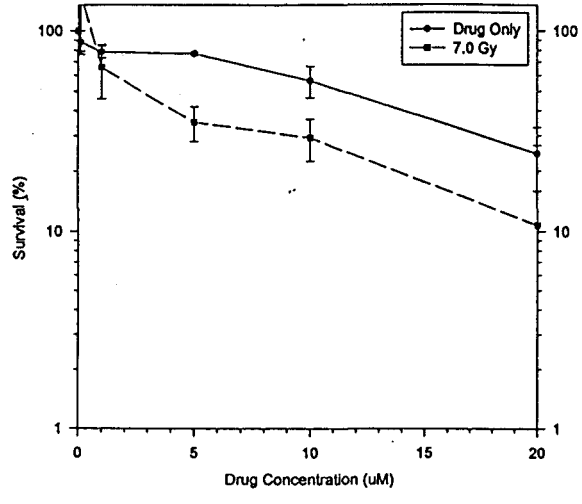
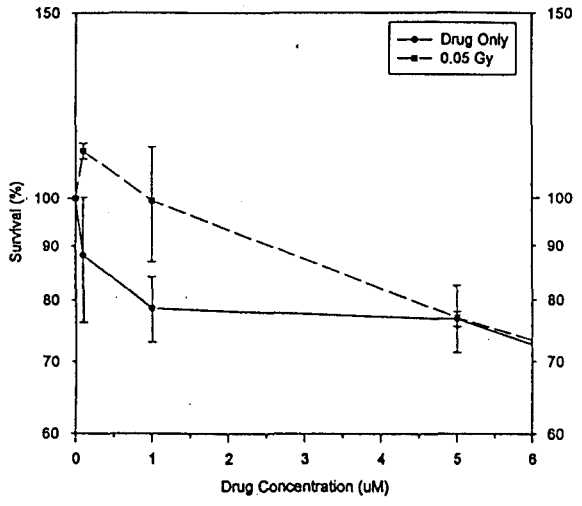


e)

Figure 16: Cisplatin survival curves for SCC-25 cells treated with radiation 60 minutes before cisplatin. The curves for a) 0.05 Gy, b) 0.25 Gy, c) 1.0 Gy, d) 2.5 Gy and e) 7.0 Gy radiation pretreatment are shown in relation to the cisplatin survival curve without radiation pretreatment. The curves are plotted for cisplatin concentrations ranging from 0.1 to 20 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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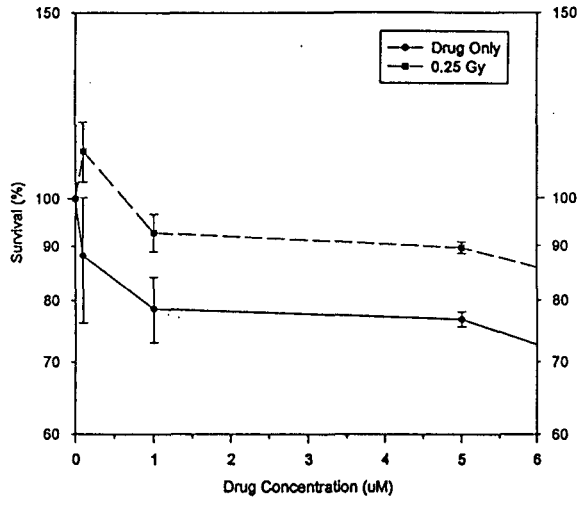

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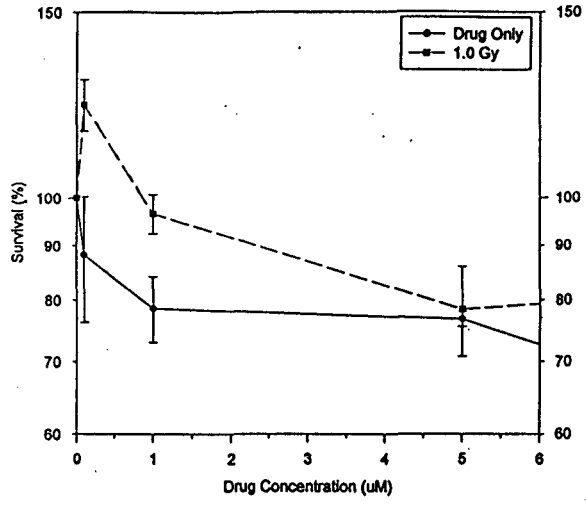

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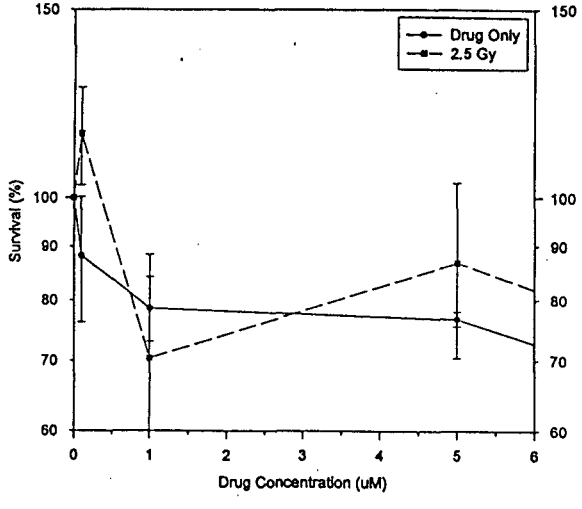

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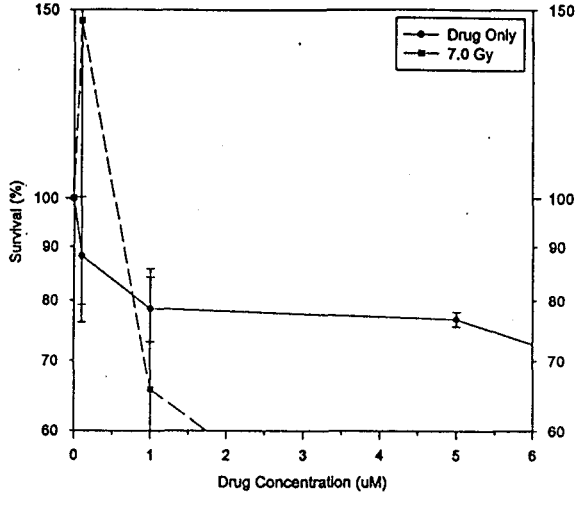


e)

Figure 17: Cisplatin survival curves for SCC-25 cells treated with radiation 60 minutes before cisplatin. The curves for a) 0.05 Gy, b) 0.25 Gy, c) 1.0 Gy, d) 2.5 Gy and e) 7.0 Gy radiation pretreatment are shown in relation to the cisplatin survival curve without radiation pretreatment. The curves are plotted for cisplatin concentrations ranging from 0.1 to 5 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*

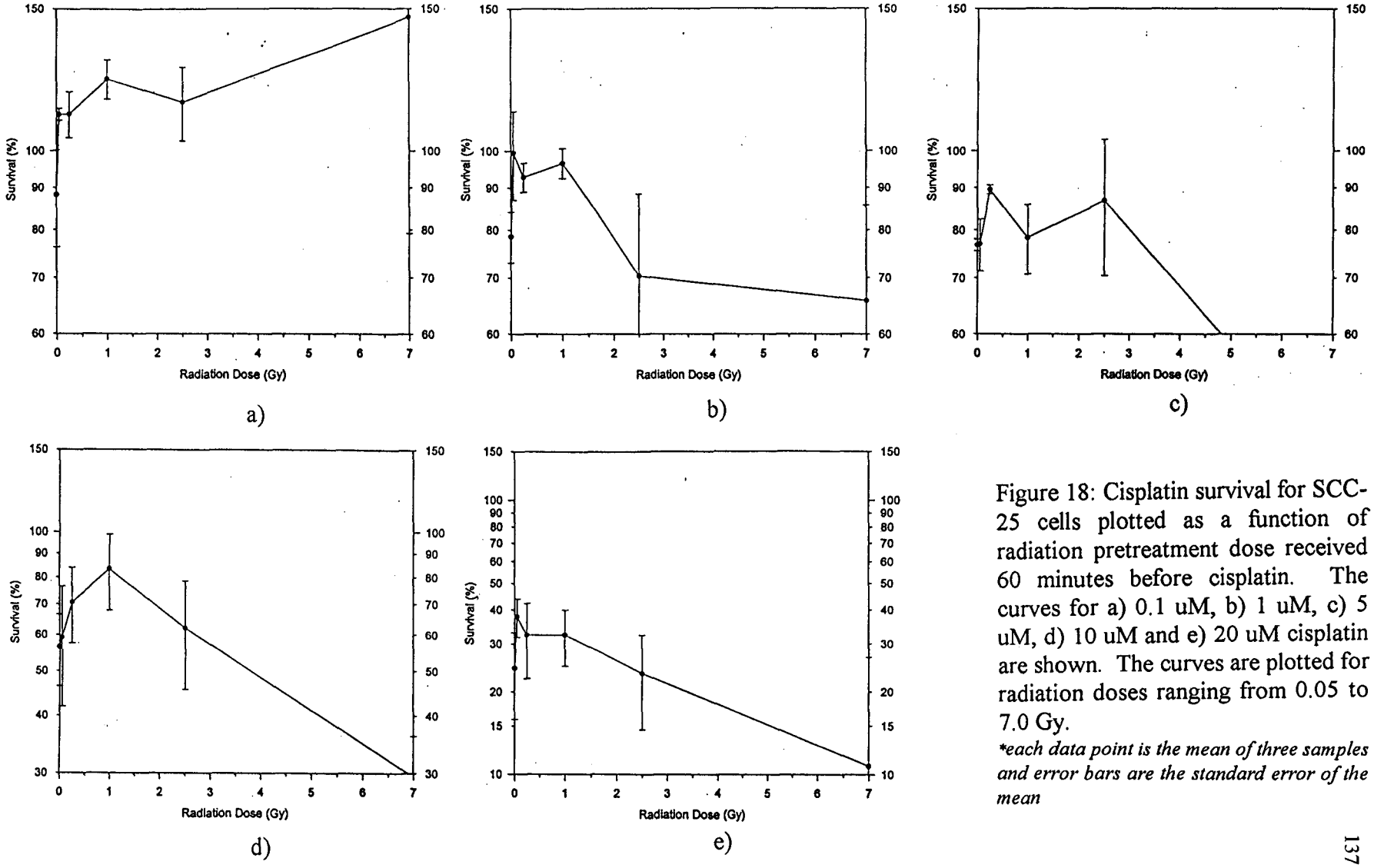


Figure 18: Cisplatin survival for SCC-25 cells plotted as a function of radiation pretreatment dose received 60 minutes before cisplatin. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin are shown. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*

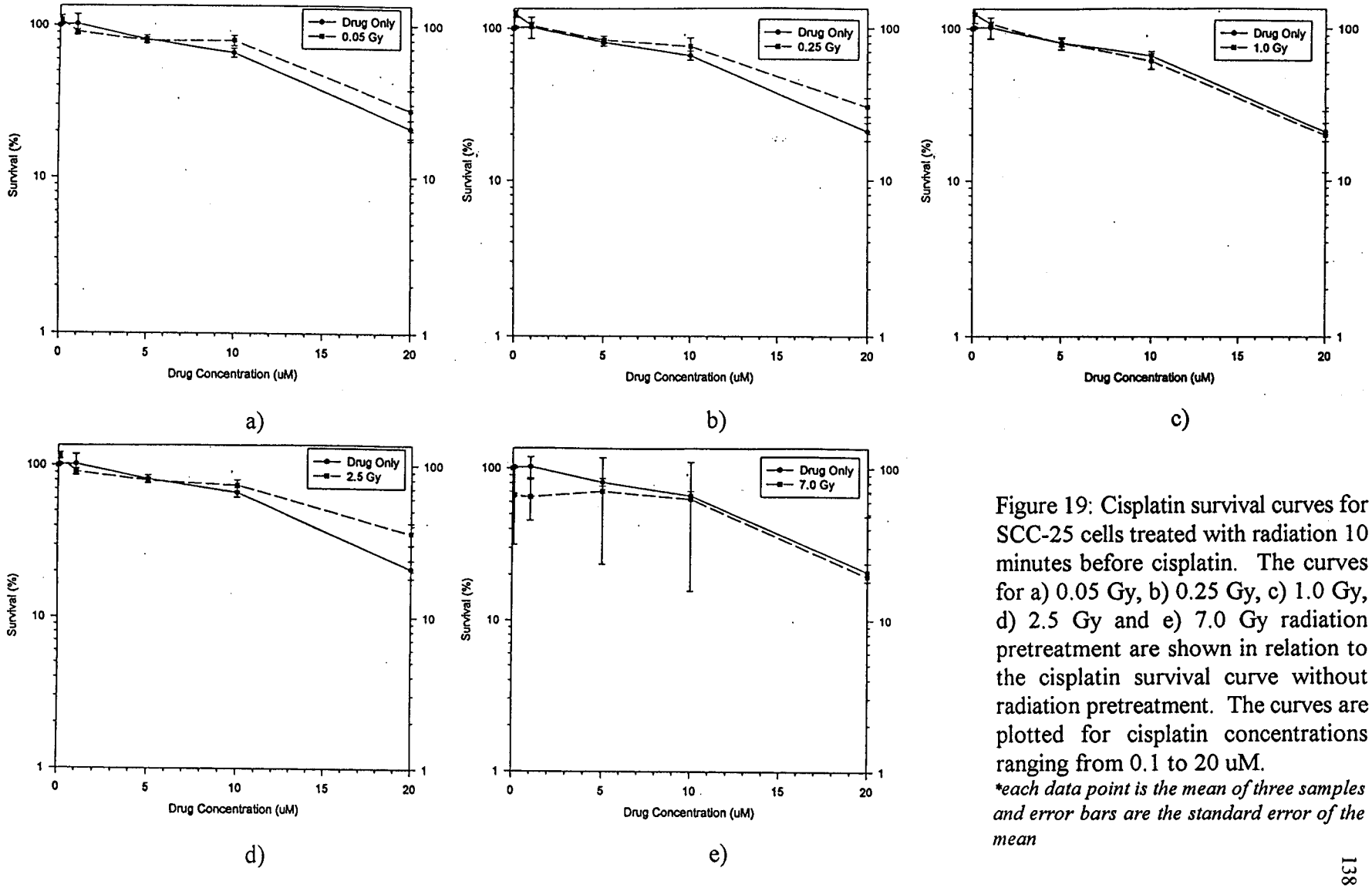


Figure 19: Cisplatin survival curves for SCC-25 cells treated with radiation 10 minutes before cisplatin. The curves for a) 0.05 Gy, b) 0.25 Gy, c) 1.0 Gy, d) 2.5 Gy and e) 7.0 Gy radiation pretreatment are shown in relation to the cisplatin survival curve without radiation pretreatment. The curves are plotted for cisplatin concentrations ranging from 0.1 to 20 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*

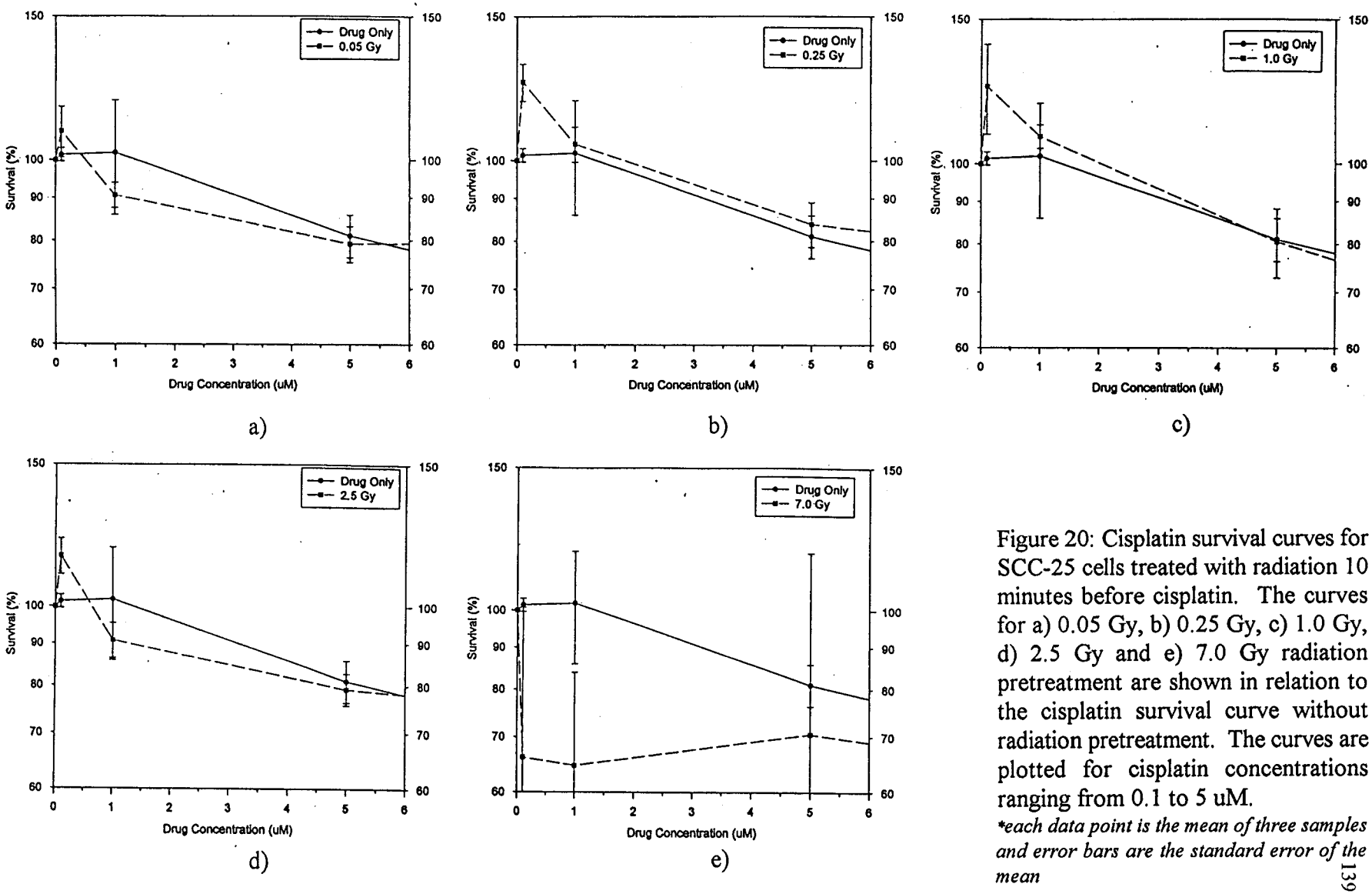


Figure 20: Cisplatin survival curves for SCC-25 cells treated with radiation 10 minutes before cisplatin. The curves for a) 0.05 Gy, b) 0.25 Gy, c) 1.0 Gy, d) 2.5 Gy and e) 7.0 Gy radiation pretreatment are shown in relation to the cisplatin survival curve without radiation pretreatment. The curves are plotted for cisplatin concentrations ranging from 0.1 to 5 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*

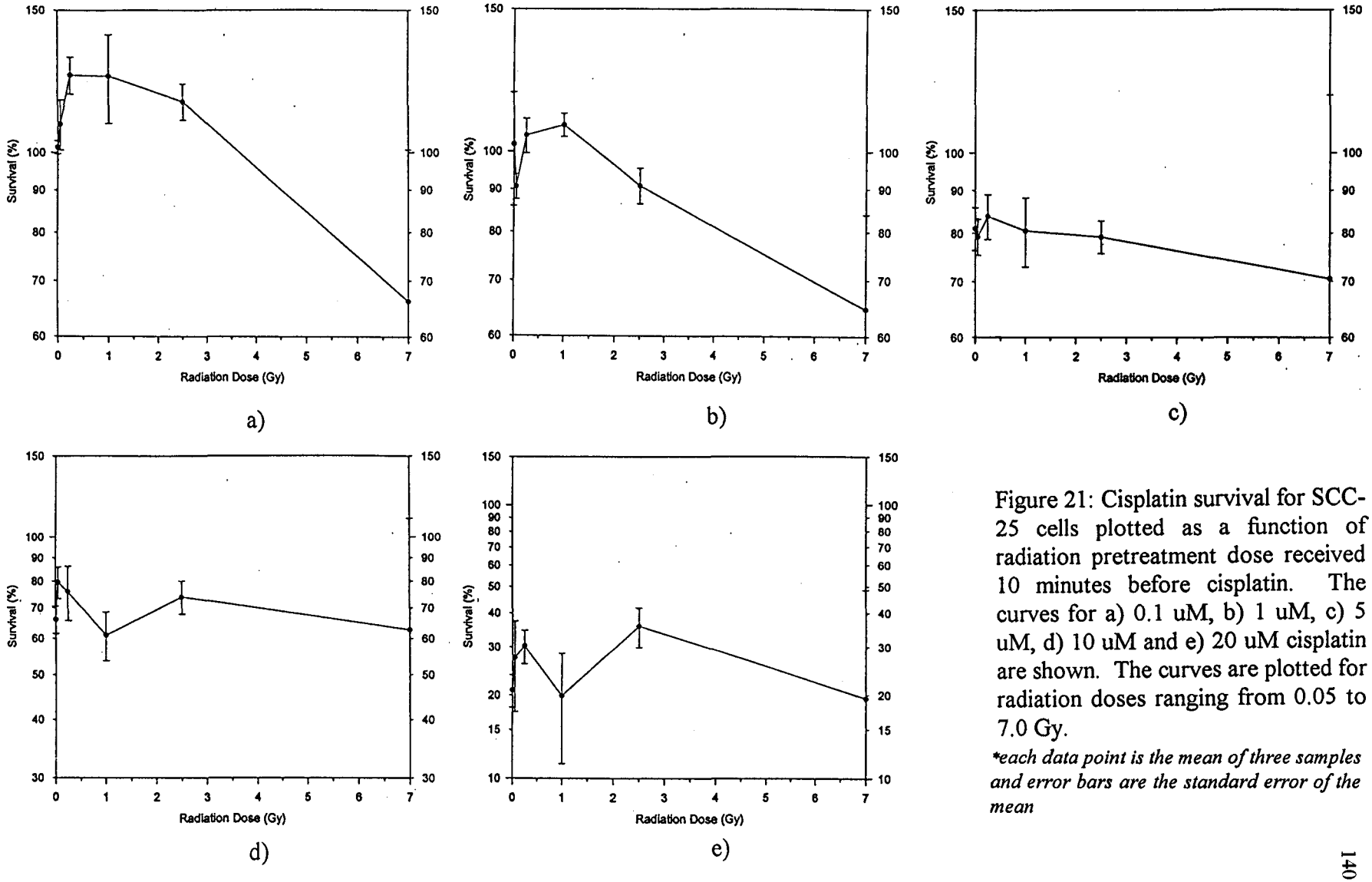
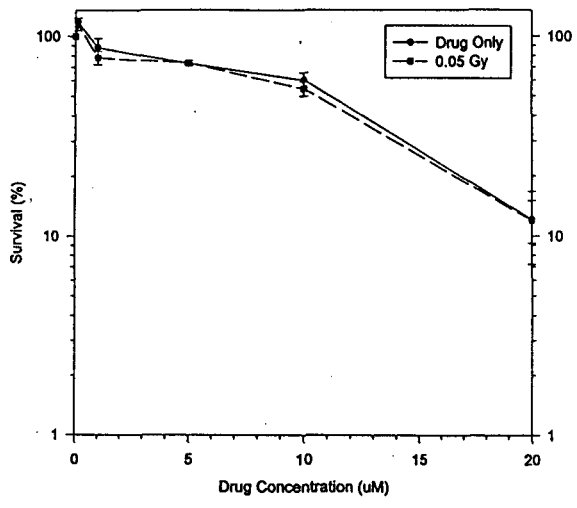


Figure 21: Cisplatin survival for SCC-25 cells plotted as a function of radiation pretreatment dose received 10 minutes before cisplatin. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin are shown. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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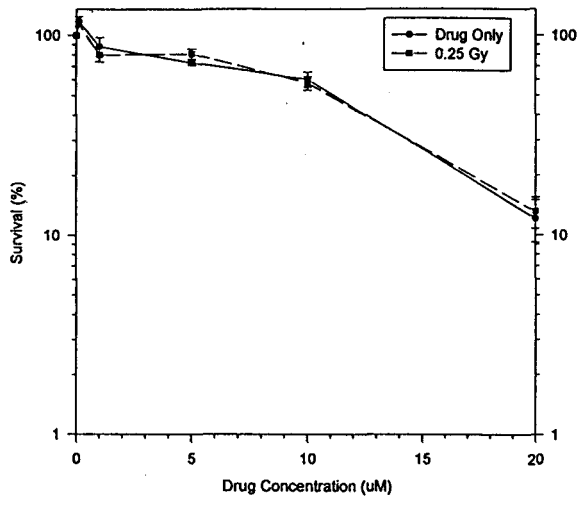

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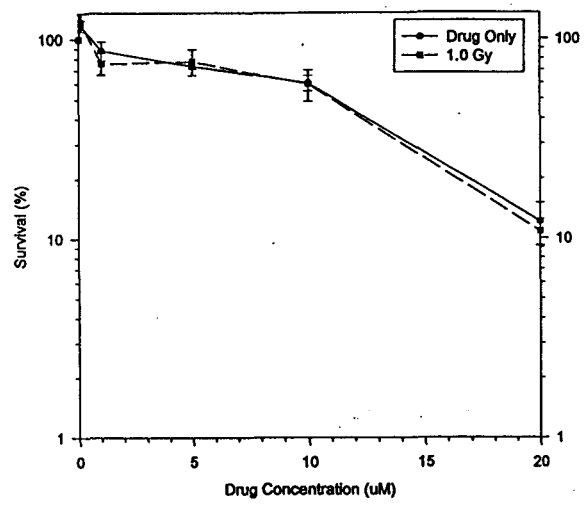

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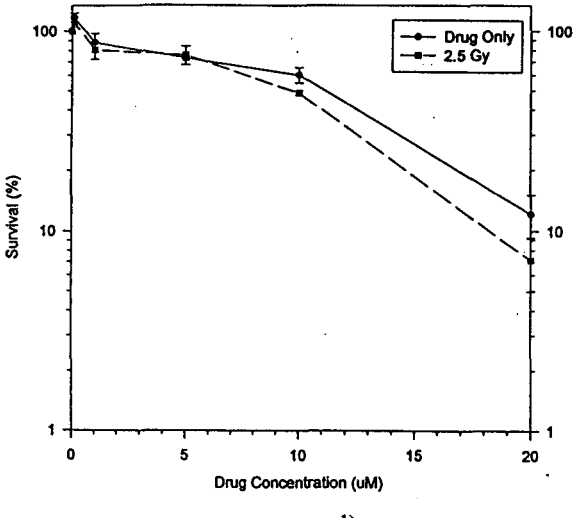

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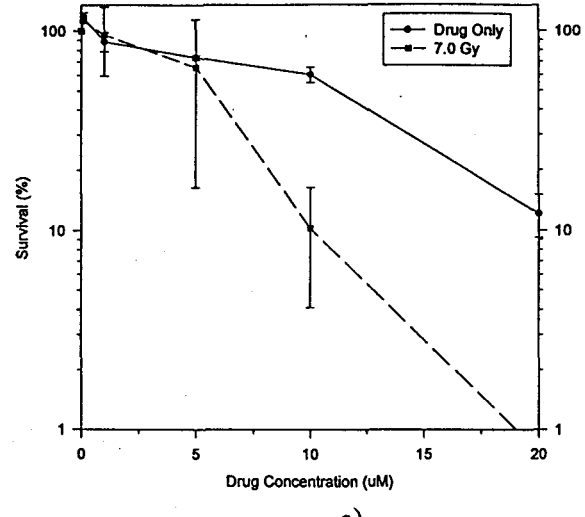
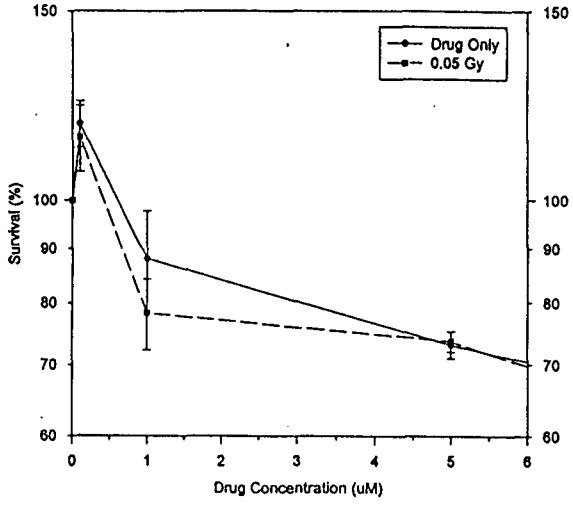


e)

Figure 22: Cisplatin survival curves for SCC-25 cells treated with radiation 10 minutes after cisplatin. The curves for a) 0.05 Gy, b) 0.25 Gy, c) 1.0 Gy, d) 2.5 Gy and e) 7.0 Gy radiation treatment are shown in relation to the cisplatin survival curve without radiation treatment. The curves are plotted for cisplatin concentrations ranging from 0.1 to 20 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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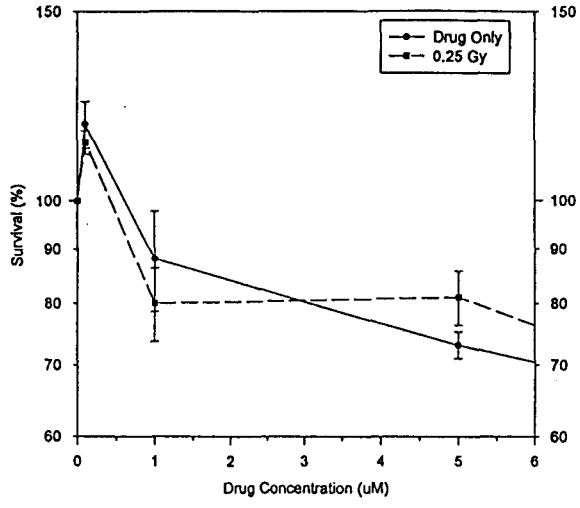

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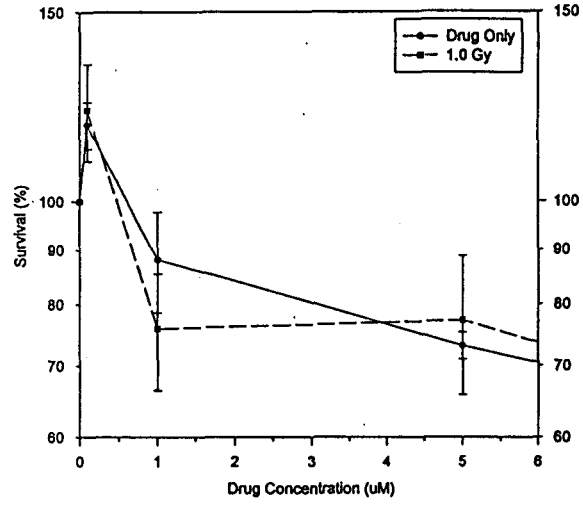

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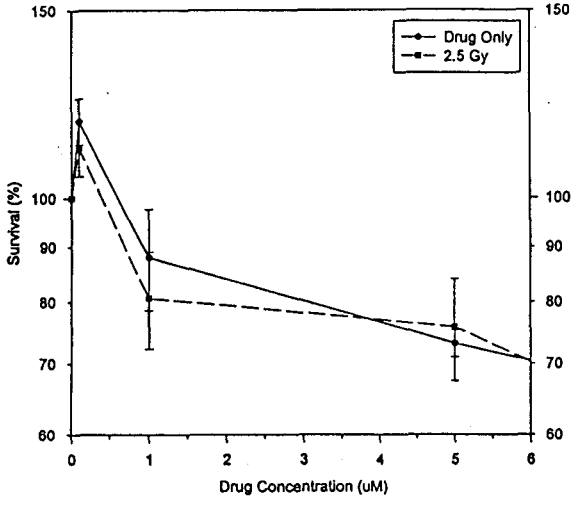

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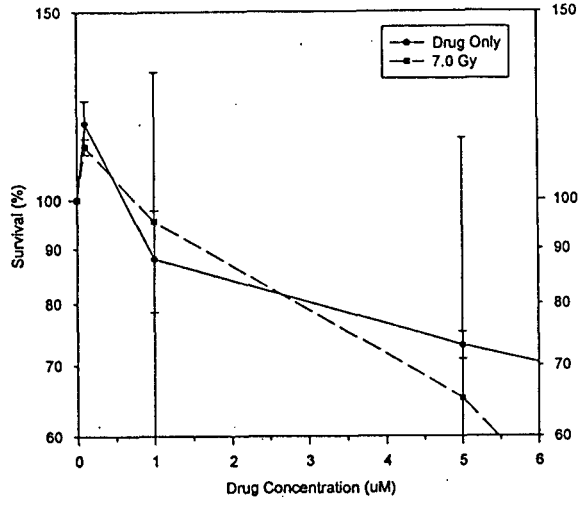
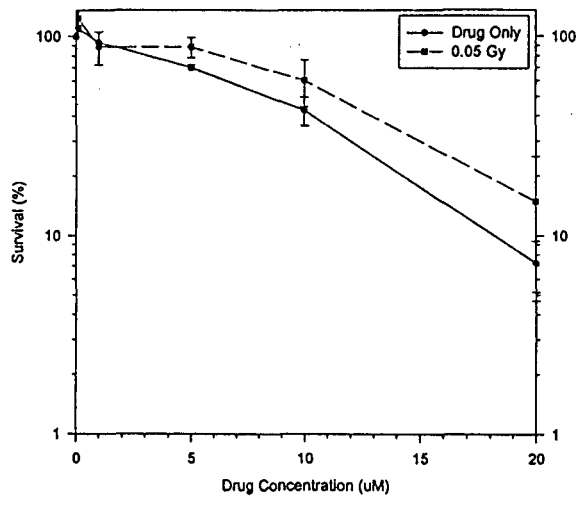


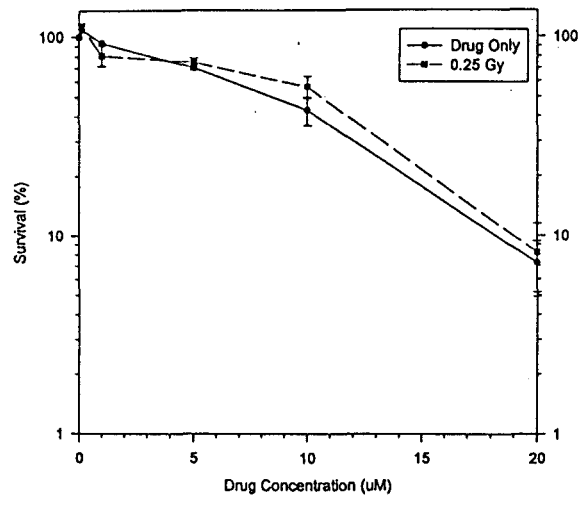
e)

Figure 23: Cisplatin survival curves for SCC-25 cells treated with radiation 10 minutes after cisplatin. The curves for a) 0.05 Gy, b) 0.25 Gy, c) 1.0 Gy, d) 2.5 Gy and e) 7.0 Gy radiation treatment are shown in relation to the cisplatin survival curve without radiation treatment. The curves are plotted for cisplatin concentrations ranging from 0.1 to 5 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*



a)



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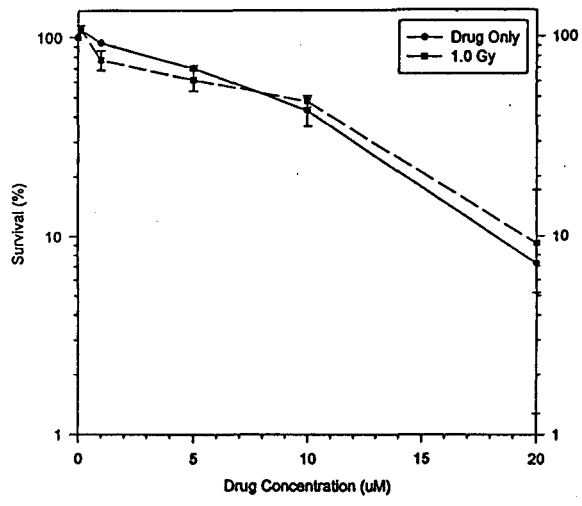

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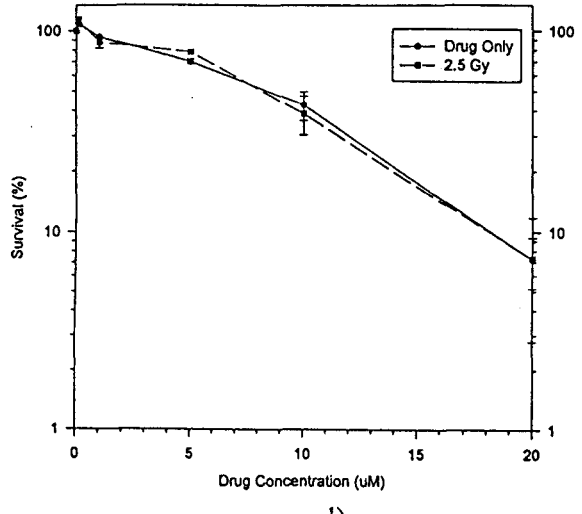

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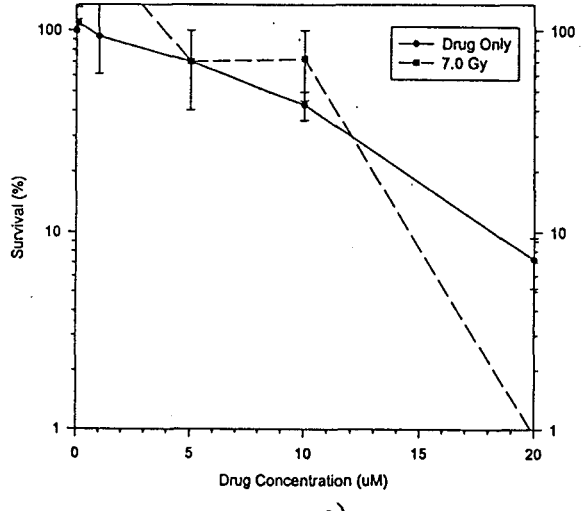


e)

Figure 24: Cisplatin survival curves for SCC-25 cells treated with radiation 60 minutes after cisplatin. The curves for a) 0.05 Gy, b) 0.25 Gy, c) 1.0 Gy, d) 2.5 Gy and e) 7.0 Gy radiation treatment are shown in relation to the cisplatin survival curve without radiation treatment. The curves are plotted for cisplatin concentrations ranging from 0.1 to 20 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*

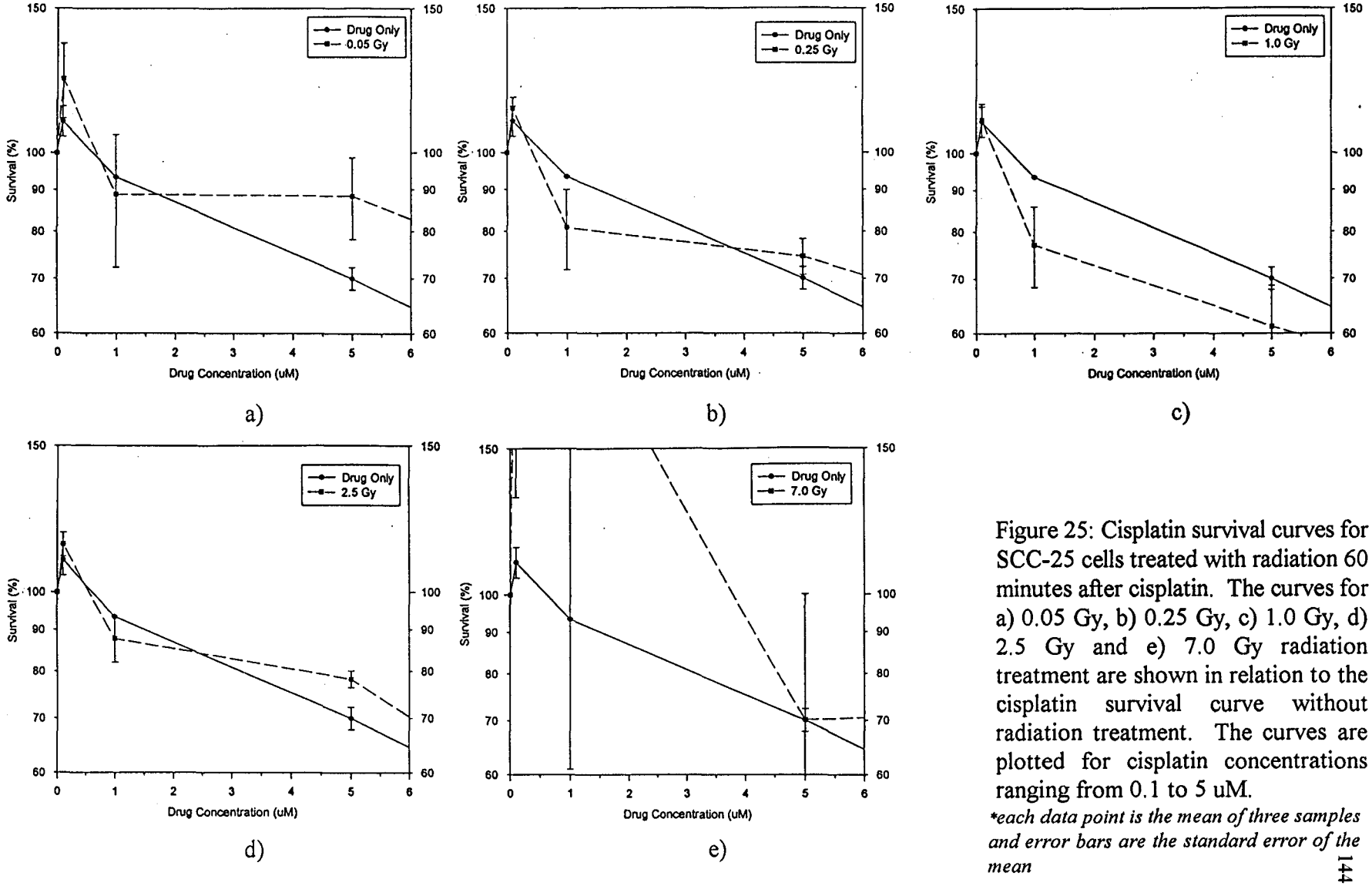
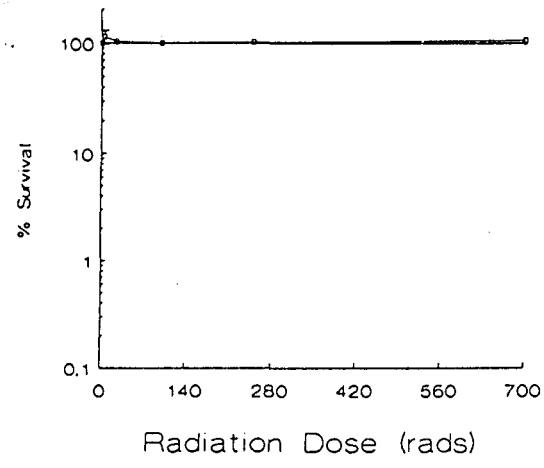


Figure 25: Cisplatin survival curves for SCC-25 cells treated with radiation 60 minutes after cisplatin. The curves for a) 0.05 Gy, b) 0.25 Gy, c) 1.0 Gy, d) 2.5 Gy and e) 7.0 Gy radiation treatment are shown in relation to the cisplatin survival curve without radiation treatment. The curves are plotted for cisplatin concentrations ranging from 0.1 to 5 uM.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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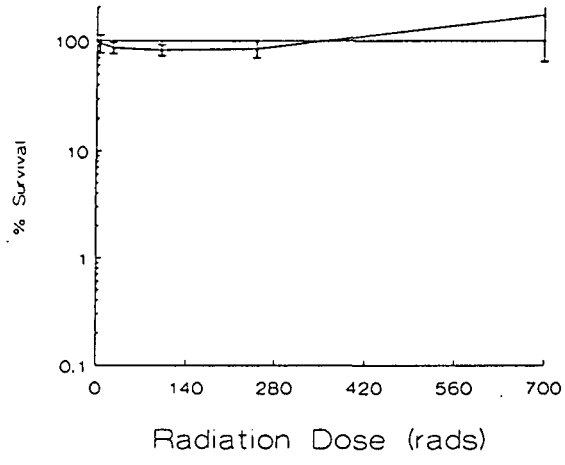

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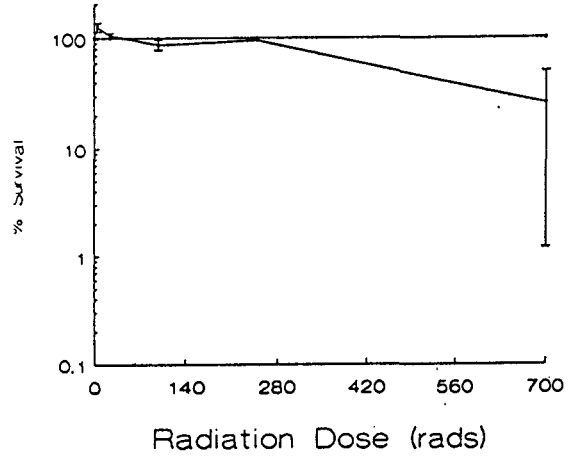

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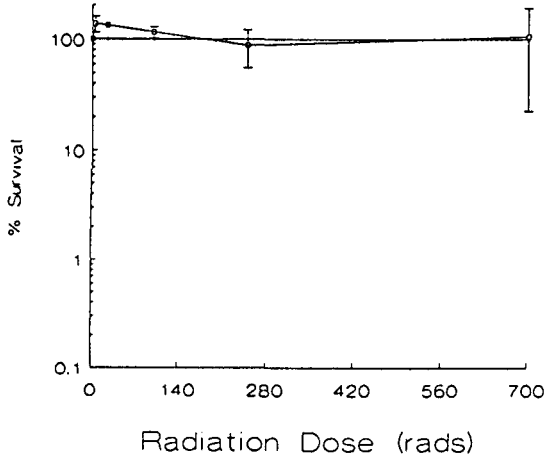

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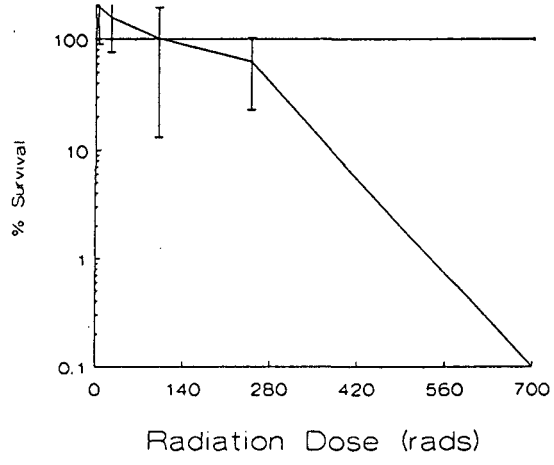
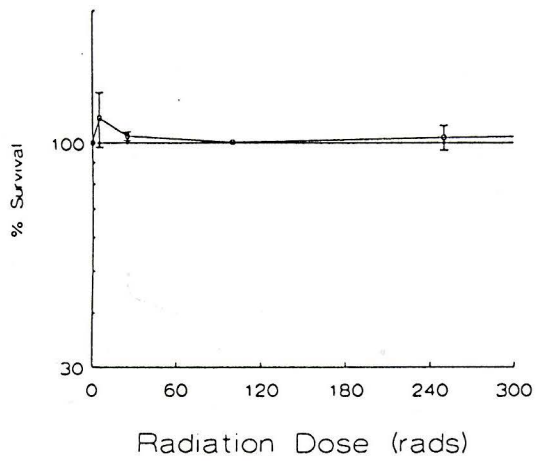


e)

Figure 26: Survival curves for SCC-25 cells treated with cisplatin 60 minutes before radiation, corrected for both cisplatin and radiation treatment. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin are shown in relation to the line of additivity at 100 per cent survival. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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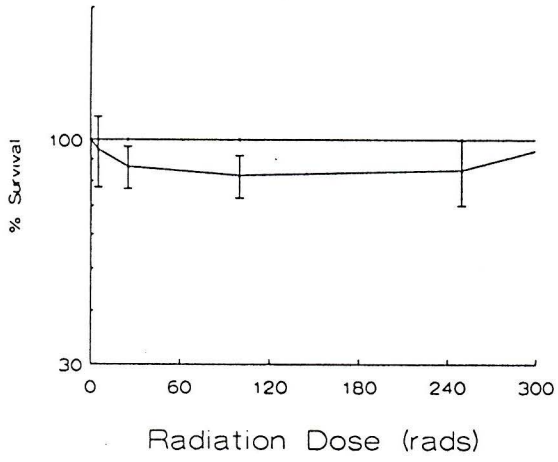

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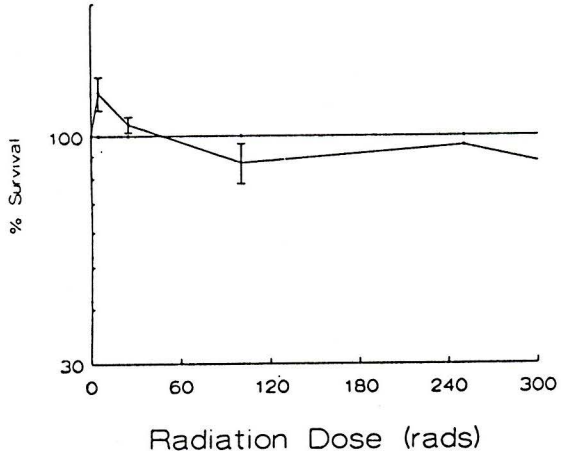

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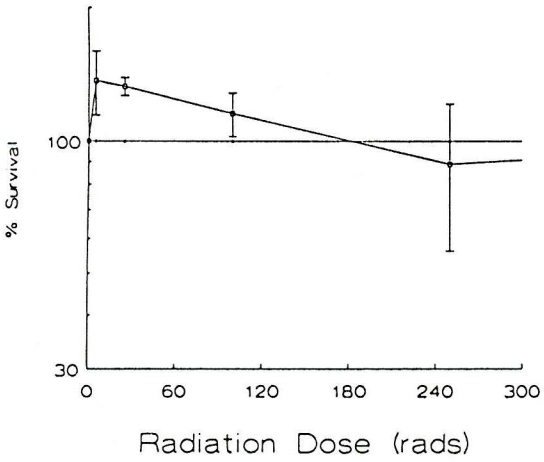

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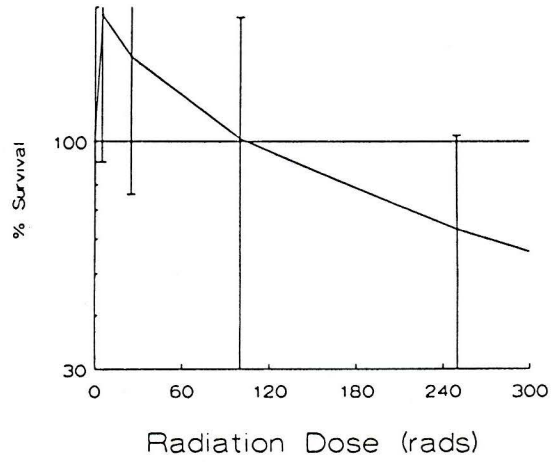
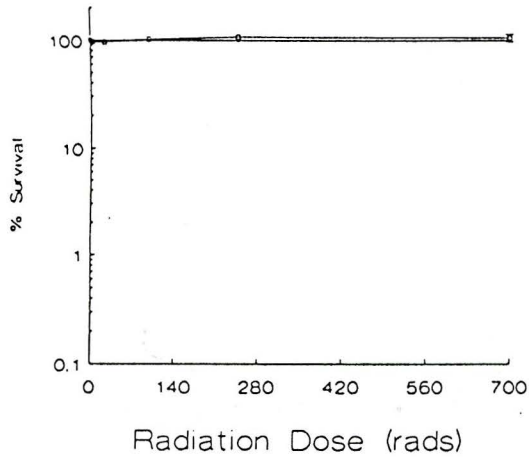


e)

Figure 27: Survival curves for SCC-25 cells treated with cisplatin 60 minutes before radiation, corrected for both cisplatin and radiation treatment. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin are shown in relation to the line of additivity at 100 per cent survival. The curves are plotted for radiation doses ranging from 0.05 to 2.5 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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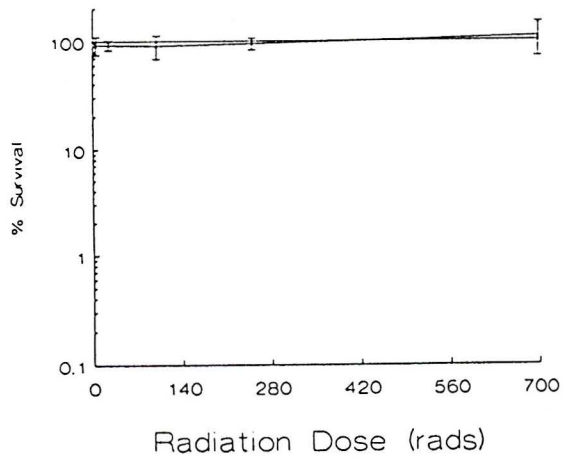

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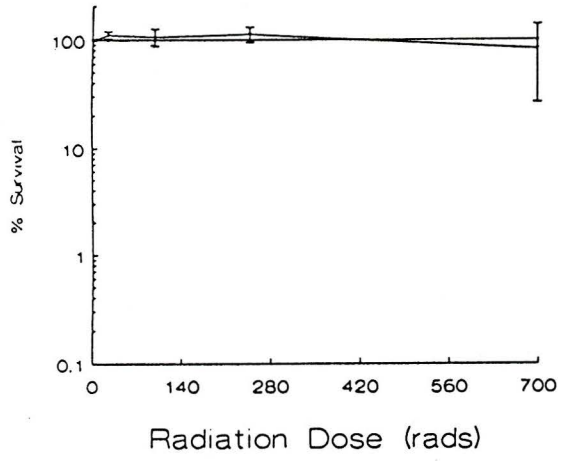

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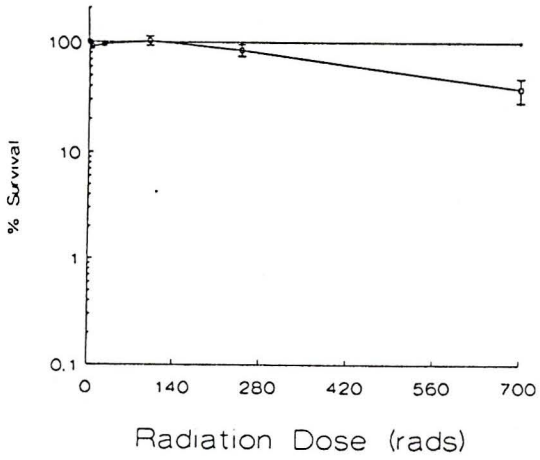

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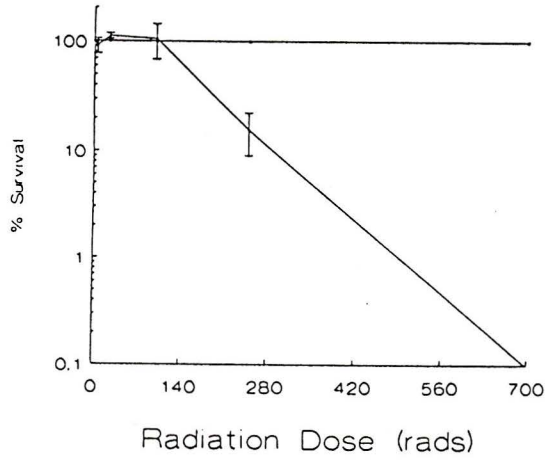
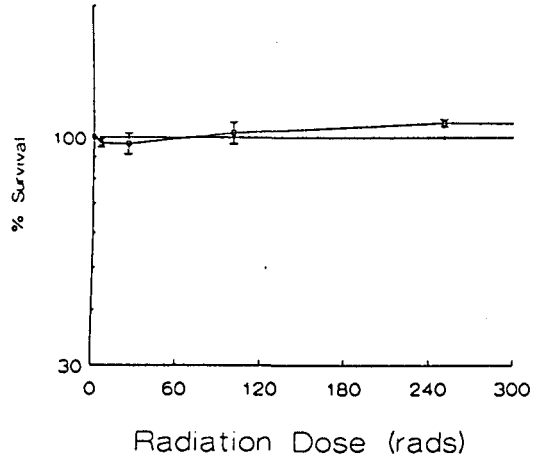


e)

Figure 28: Survival curves for SCC-25 cells treated with cisplatin 10 minutes before radiation, corrected for both cisplatin and radiation treatment. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin are shown in relation to the line of additivity at 100 per cent survival. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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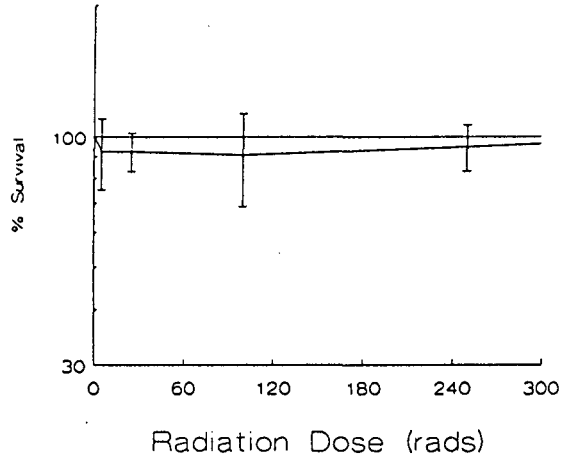

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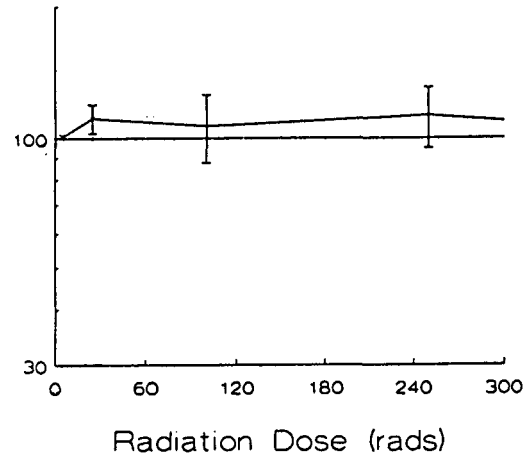

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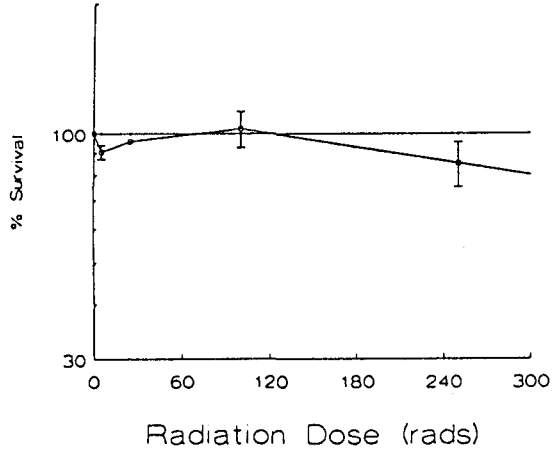

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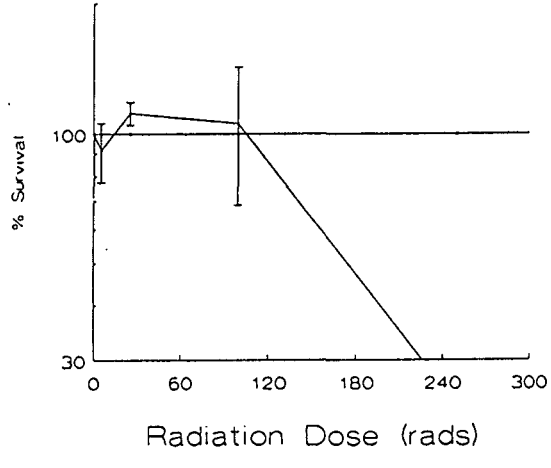
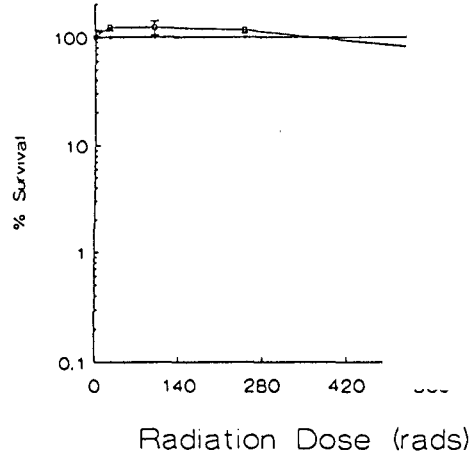


e)

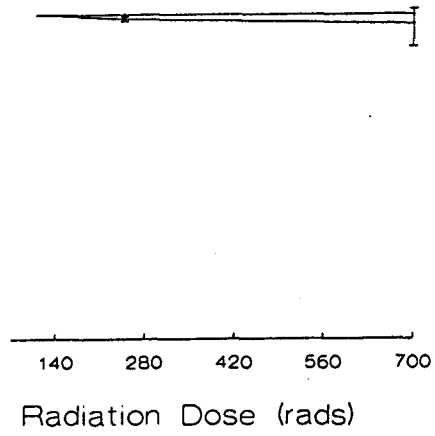
Figure 29: Survival curves for SCC-25 cells treated with cisplatin 10 minutes before radiation, corrected for both cisplatin and radiation treatment. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin are shown in relation to the line of additivity at 100 per cent survival. The curves are plotted for radiation doses ranging from 0.05 to 2.5 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



a)

b)



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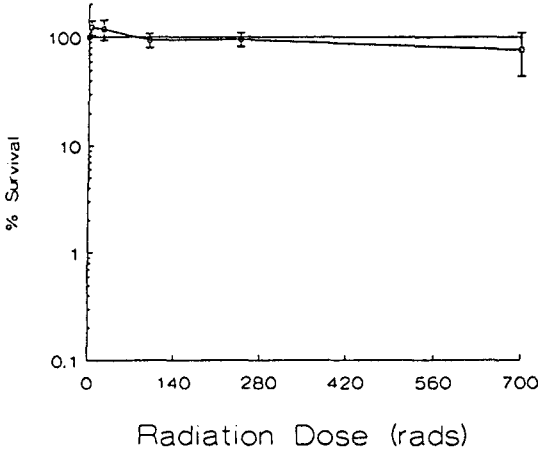

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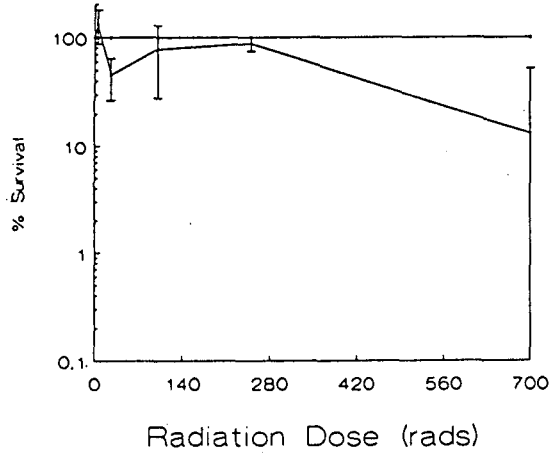
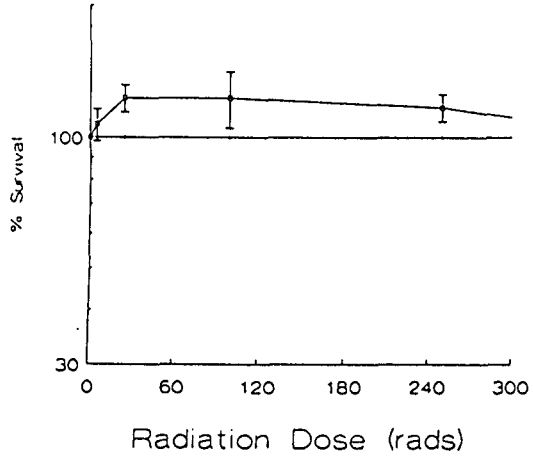


e)

Figure 30: Survival curves for SCC-25 cells treated with cisplatin 10 minutes after radiation, corrected for both cisplatin and radiation treatment. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin are shown in relation to the line of additivity at 100 per cent survival. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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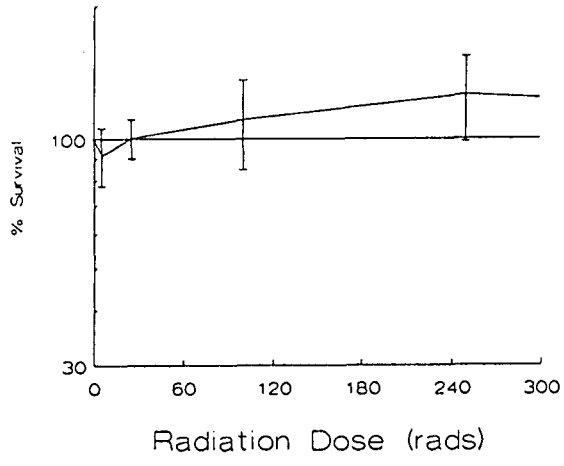

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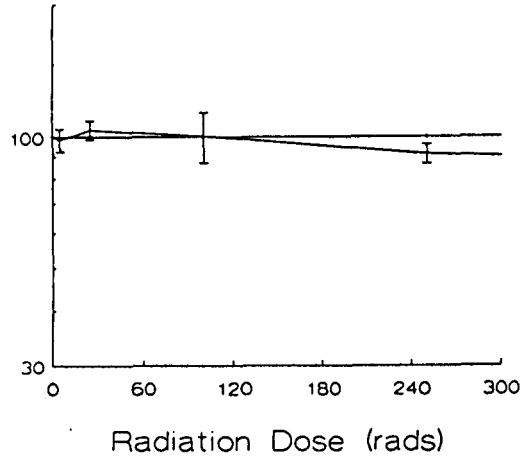

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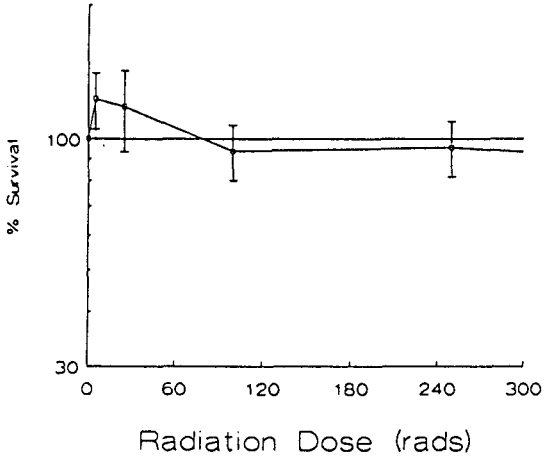

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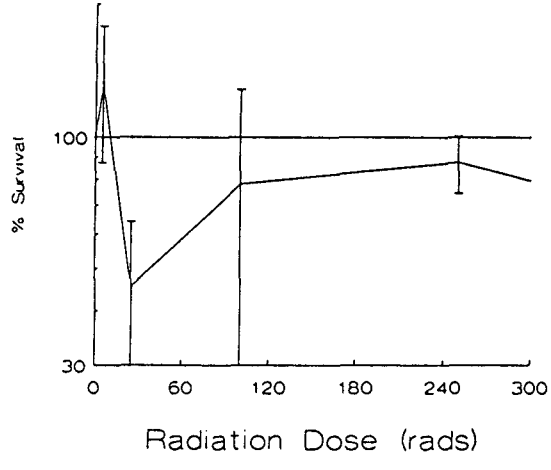
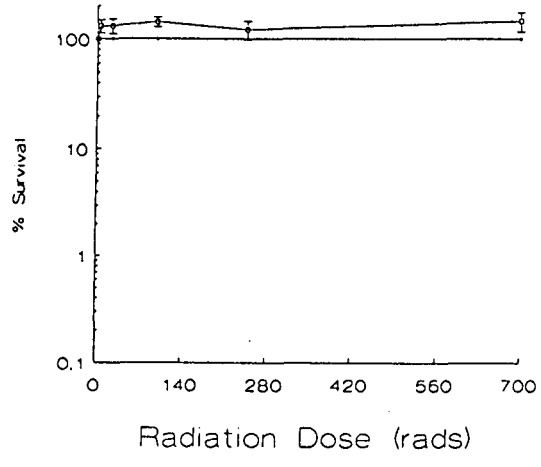


e)

Figure 31: Survival curves for SCC-25 cells treated with cisplatin 10 minutes after radiation, corrected for both cisplatin and radiation treatment. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin are shown in relation to the line of additivity at 100 per cent survival. The curves are plotted for radiation doses ranging from 0.05 to 2.5 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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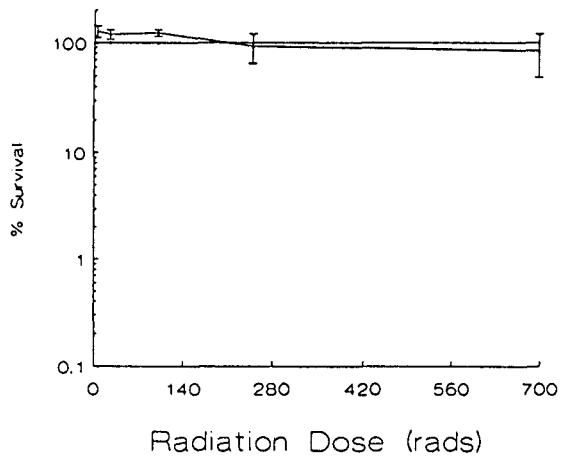

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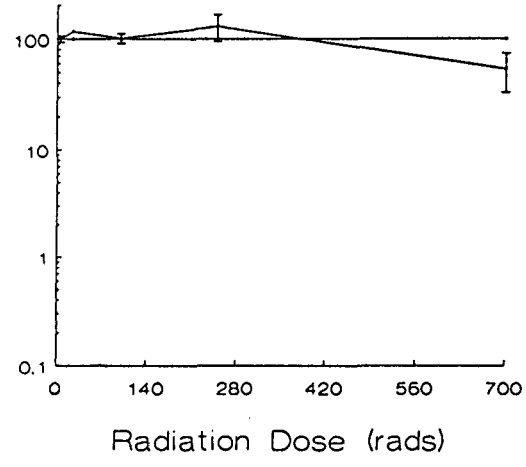

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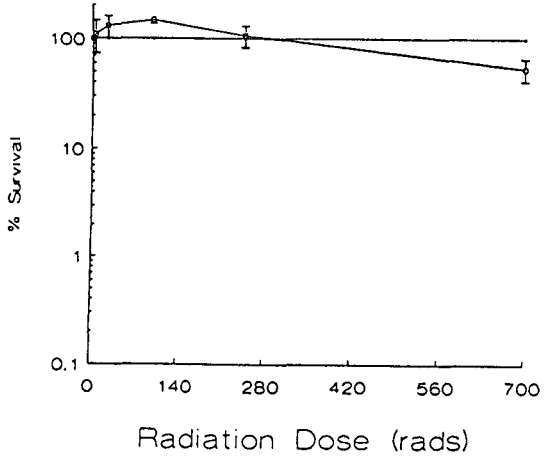

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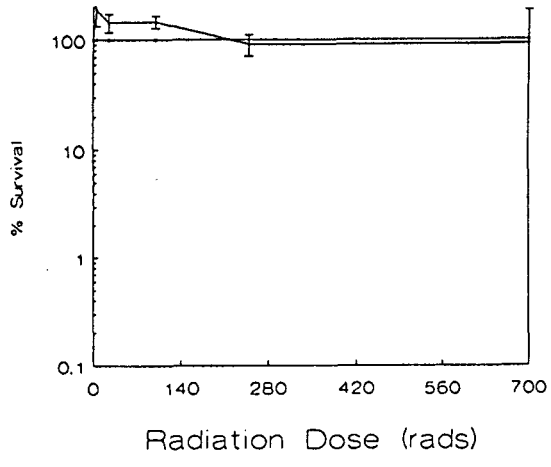
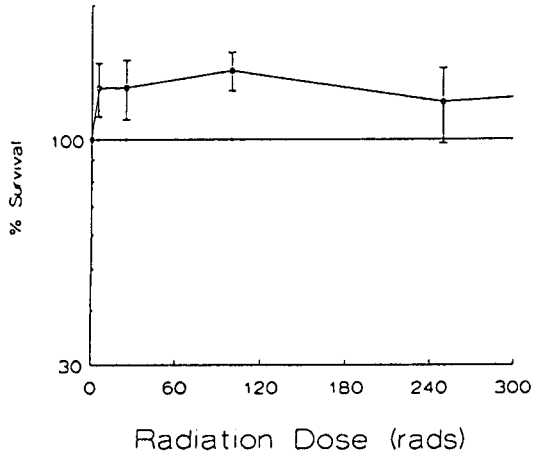


e)

Figure 32: Survival curves for SCC-25 cells treated with cisplatin 60 minutes after radiation, corrected for both cisplatin and radiation treatment. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin are shown in relation to the line of additivity at 100 per cent survival. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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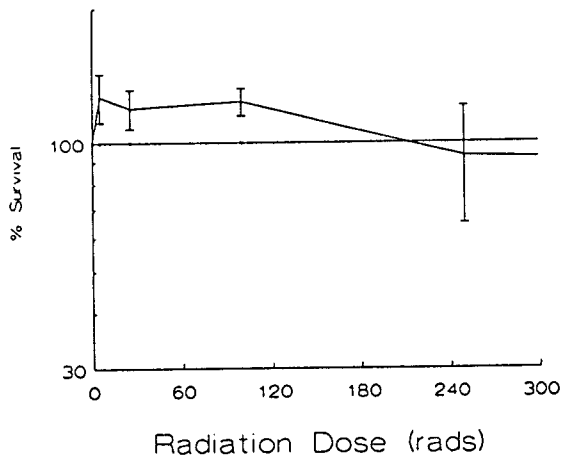

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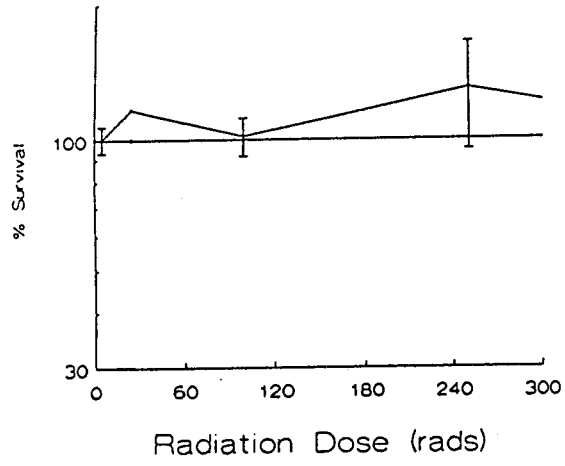

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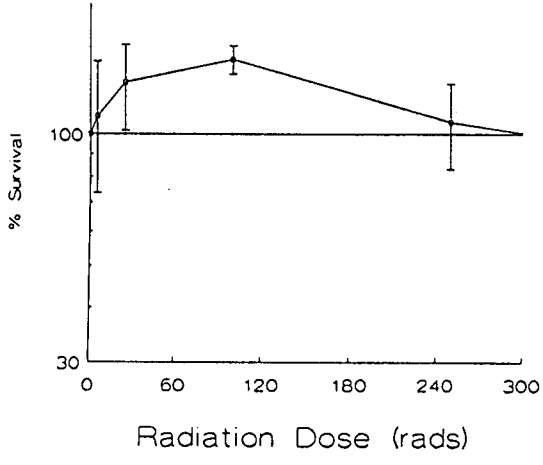

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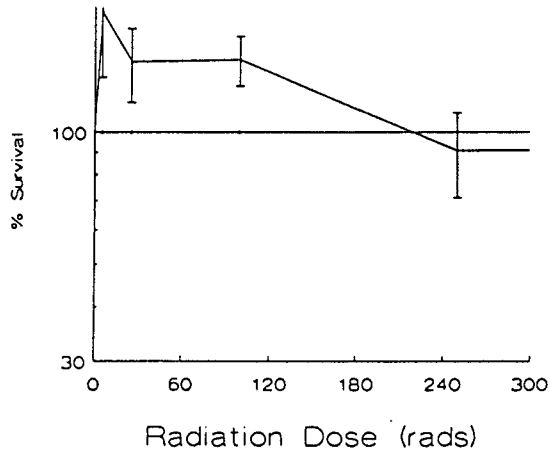
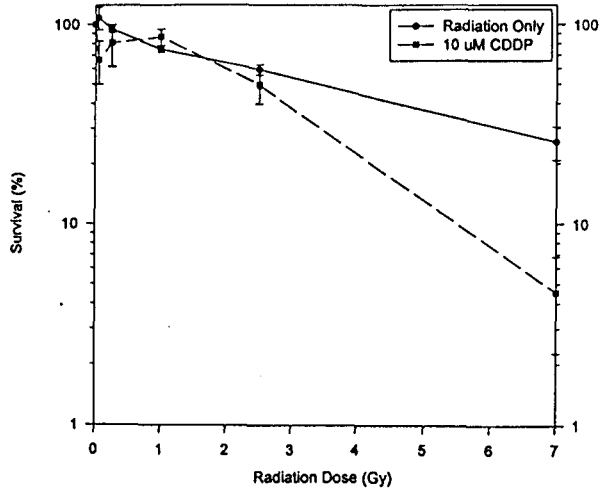


e)

Figure 33: Survival curves for SCC-25 cells treated with cisplatin 60 minutes after radiation, corrected for both cisplatin and radiation treatment. The curves for a) 0.1 uM, b) 1 uM, c) 5 uM, d) 10 uM and e) 20 uM cisplatin are shown in relation to the line of additivity at 100 per cent survival. The curves are plotted for radiation doses ranging from 0.05 to 2.5 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



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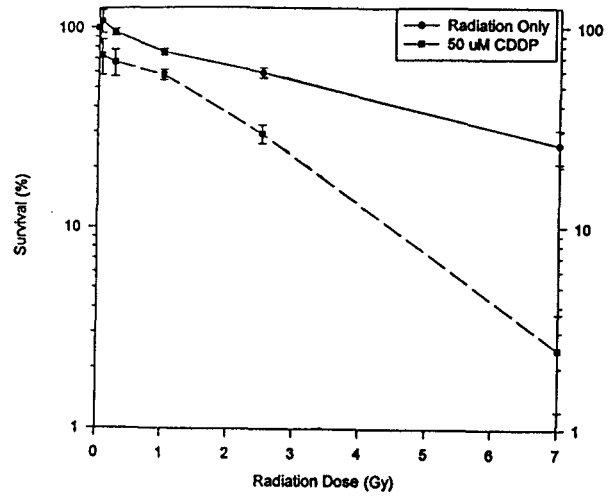
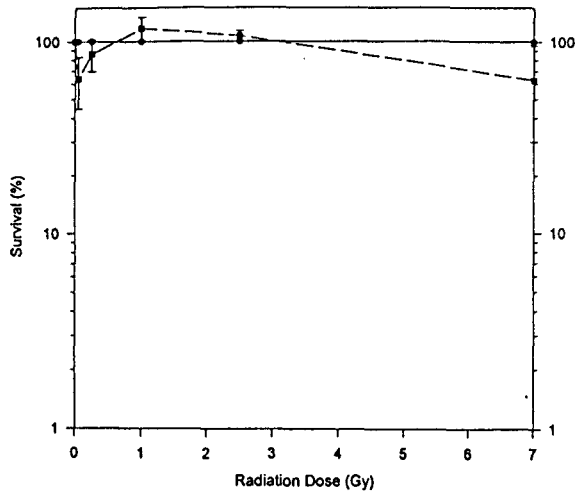


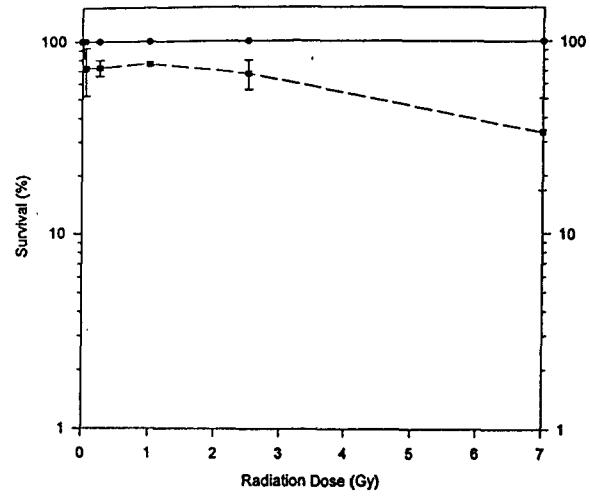
b)

Figure 34: Radiation survival curves for SCC-25/CP cells treated with cisplatin 60 minutes after radiation. The curves for a) 10 uM and b) 50 uM cisplatin are shown in relation to the radiation survival curve without cisplatin treatment. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



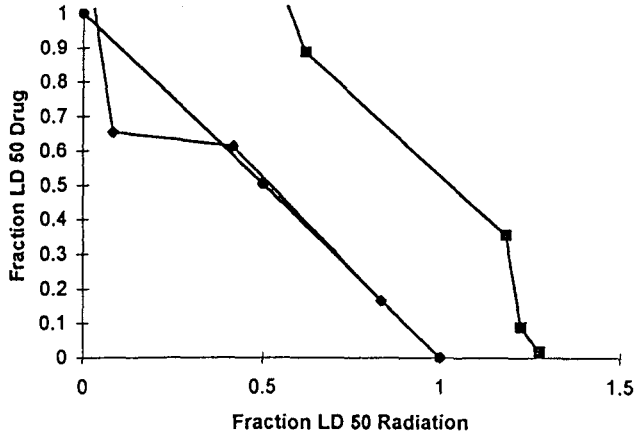
a)



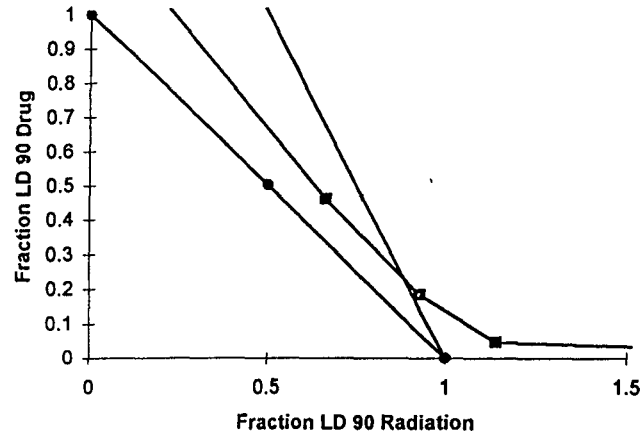
b)

Figure 35: Survival curves for SCC-25/CP cells treated with cisplatin 60 minutes after radiation, corrected for both cisplatin and radiation treatment. The curves for a) 10 uM and b) 50 uM cisplatin are shown in relation to the line of additivity at 100 per cent survival. The curves are plotted for radiation doses ranging from 0.05 to 7.0 Gy.

**each data point is the mean of three samples and error bars are the standard error of the mean*



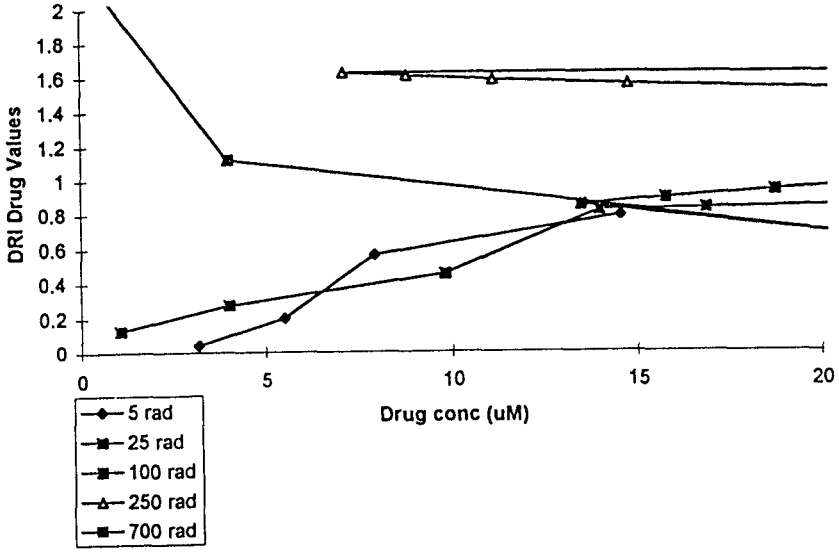
a)



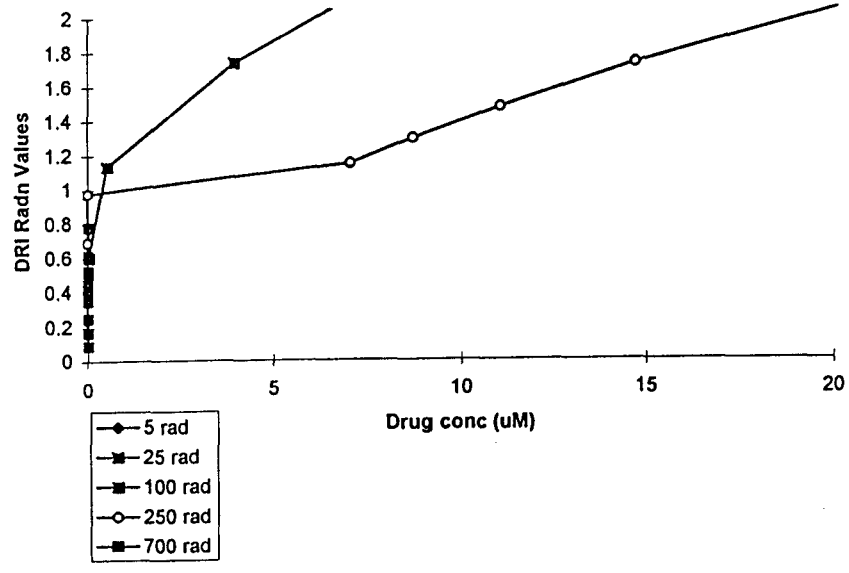
b)

Figure 36: Isobolograms for SCC-25 cells treated with cisplatin 60 minutes after radiation. Isobolograms are expressed as a function of the lethal dose (LD) of radiation required to kill a) 50 per cent and b) 90 per cent of cells. The observed data points (▪) are plotted relative to the expected additive line (•) and the modified additive line (◆).

**each data point is the mean of three samples and error bars are the standard error of the mean*



a)



b)

Figure 37: Dose reduction indices (DRIs) for SCC-25 cells treated with cisplatin 60 minutes after radiation. DRI values for a) cisplatin and b) radiation are plotted as a function of cisplatin concentration.
**each data point is the mean of three samples and error bars are the standard error of the mean*

Discussion

When cisplatin was given first , followed by radiation ten minutes later, all combination treatments produced the same surviving fraction as radiation alone, regardless of the radiation doses or drug concentrations used. This was concluded because there were no significant differences identified for any combination points compared to radiation using the t-test. There was also no pattern in the differences between the combinations and radiation treatment. Combination treatment increased cell kill relative to drug alone for a few combinations in which high drug concentrations or high radiation doses were used. The observation of a response for these few combinations is based on the significant values from the t-test which existed only for some of the combinations that used an LD 50 of cisplatin or an LD 90 of radiation. However, the observed synergy was not consistent on the dose and concentration range since there were high drug concentrations for which no synergy was found. Therefore, it is concluded that drug followed by radiation at 10 minutes results in additive cell kill. There is a suggestion of synergy for higher drug concentrations and radiation doses, but this experiment does not provide conclusive evidence that synergy exists.

When drug was given first and radiation 60 minutes later, there were only two combinations which were statistically different from drug or radiation only. According to the t values, there was no consistency in the direction of difference. Therefore,

combination cisplatin and radiation in this schedule was observed to result in additive cell kill only, regardless of the drug concentration or radiation dose used.

When radiation was given first followed by drug 10 minutes later, several combinations were found to statistically differ from the radiation only line. Only combinations in which the lowest drug concentration of 0.1 uM was used, seem to consistently result in an increase in survival compared to radiation alone. It should be noted that only combinations using 0.1 uM (≤ LD 10) were statistically different from the radiation only line. This difference was consistently in the direction of increasing survival. Therefore, a slight antagonism has been identified as a pattern in the response of cells to combination treatment at low drug concentration. No combinations were significantly different from drug alone and no pattern was found for combinations compared to drug alone.

Many combinations were found to differ significantly from radiation alone when radiation was administered first and followed by drug 60 minutes later. There was also a consistent pattern in the differences for low radiation doses and the two lowest cisplatin concentrations used (≤ LD 10). The differences were found to be in the direction of increasing survival compared to radiation alone. A difference consistent with this pattern was also found for low radiation doses and an LD 90 of cisplatin. Combinations were also found to differ significantly from the drug only line for low drug concentrations and low radiation doses. Again the difference was found in the direction of increased survival. Therefore, a moderate antagonism exists for cisplatin and radiation in combination for low radiation doses and low drug concentrations (≤ LD 10 for radiation and cisplatin).

The experiments were conducted without fixing the oxygen level of the samples, so it may be that cisplatin is only a hypoxic radiosensitizer in this cell line. Hypoxia has been shown to be a condition for cisplatin radiosensitization in several cell lines[81, 78, 79, 82, 83]. The mechanism for hypoxic radiosensitization by cisplatin is not known. In fact, hypoxic radiosensitization seems counter-intuitive given that the presence of oxygen in tissues during radiation is known to increase radiosensitization[124]. This is referred to as the oxygen effect, and is more prominent when low LET radiation, such as x-rays or γ-rays, is used[124]. The clinical use of combination cisplatin-radiation treatment has been advocated on the basis that cisplatin is a hypoxic radiosensitizer[81, 78, 79, 82, 83], and that hypoxic cells are commonly found in tumor masses. Hypoxic cells are normally difficult to destroy because chemotherapy depends on tumor vascularization for the delivery of anti tumor drugs, and radiotherapy depends, in part, on the presence of oxygen for the generation of free radicals to produce maximum cell damage. The clinical advantage of combination cisplatin-radiation treatment, due to its ability to radiosensitize hypoxic cells, remains questionable because cisplatin is delivered via vascularization and hypoxic cells are poorly vascularized so they may not be exposed to significant concentrations of cisplatin.

The schedules for combining cisplatin and radiation were appropriate for determining the dependence of radiosensitization on cell cycle kinetics. The doubling time of the cell line is more than 24 hours, so after the first treatment, cisplatin or radiation, one would expect the cells to be in the same cell cycle phase 10 or 60 minutes later when the second treatment is given. In general, cells in G_1 and S phases are cisplatin sensitive and cells in G_2 and M phases are radiation sensitive[125, 97, 126], the cells in phases

that are resistant to the first treatment would be in the same phase which is sensitive to the second treatment. One would, therefore, expect a decrease in survival relative to either agent alone for all four schedules.

Synergy was suggested by the results when radiation followed drug at 10 minutes, but only for relatively high doses of radiation and high drug concentrations. However, it may not be meaningful to compare the results of the current work to those expected, when no cell cycle effects were investigated for this particular cell line. Determining the cell cycle phases that are sensitive to cisplatin and radiation for this particular cell line would allow a better comparison of the current results to the cell cycle model and might elucidate the mechanisms of cisplatin-radiation interaction.

There are several studies, both experimental[127-129] and clinical[117, 118] that report only additive cell kill for various combinations of cisplatin and radiation. The effect of repair inhibition is not seen in several of the cell lines used in these studies[128]. According to the model that describes cisplatin radiosensitization in terms of inhibition of radiation-induced repair, inhibition requires an interaction between the mechanisms that repair cisplatin lesions and radiation-induced lesions[107]. The experiments for each schedule are consistent with this, assuming that the repair of cisplatin adducts and of radiation-induced lesions is still ongoing both 10 minutes and 60 minutes after the first treatment (when the second treatment is administered). This is a reasonable assumption for the repair of cisplatin damage, which is known to be repaired on a relatively long time scale described by a half-life for excision repair of 24 to 60 hours[130-132].

It is well known that double DNA strand breaks are repaired rapidly, and in most cell lines the repair is complete in 10 to 15 minutes. Single DNA strand breaks are still relatively quickly repaired with more than 50% of repair complete within one hour[133]. Of course, the rapidity of repair depends on the repair proficiency of the cell type investigated. It is hypothesized that in order for cisplatin to inhibit radiation-induced repair, lesions produced by both treatments must be present within the cell at the same time. The argument is that a bulky cisplatin-DNA adduct on one DNA strand prevents the rejoining of a single-strand break on the opposite DNA strand. The cisplatin-DNA adduct is then removed by excision repair enzymes which leaves a single-strand break. Together these two single strand breaks on opposite DNA strands, form a double strand break, which is a much more lethal lesion than single-strand breaks or cisplatin-DNA adducts alone[134,135].

It has been experimentally determined that the degree of radiosensitization is increased when cisplatin damage is present during radiation[107, 104, 136]. Although, radiosensitization has been observed for cisplatin given shortly before[104, 137] or up to 24 hours before radiation[138]. The schedules examined in this project were designed in a way that should have allowed the effect of inhibition of radiation-induced damage repair to be observed, given that repair inhibition exists in the cell line used. That is, for cisplatin given first, an inhibition in radiation-induced repair should result in synergy for the schedules in which cisplatin is given first. The relative degree of radiosensitization between these two schedules may be slightly different depending on how long it takes cisplatin to form adducts with DNA and at what rate these adducts are removed. With a half-life of 24 to 60 hours for the repair of cisplatin damage, there should be a relatively small difference between the responses for a 10 and 60 minute wait between drug and

radiation. For the two schedules in which radiation is given first, there should be a relatively large radiosensitization effect when cisplatin follows at 10 minutes, compared to cisplatin given 60 minutes later when most single and double-strand DNA breaks have been repaired.

The results that cisplatin and radiation do not interact synergystically in any of the schedules investigated, suggest that cisplatin does not inhibit radiation-induced repair in the cell line examined. This assumes that the mechanism of repair inhibition described above is correct. The ability of cisplatin to inhibit radiation-induced repair in the cell line used, should be investigated. Such a study would help determine the role of radiation-induced damage repair in the response to combination cisplatin and radiation treatment. That is, if cisplatin does not produce repair inhibition in split-dose studies using this cell line, and no synergy is found, as determined by the current work, this provides support that repair inhibition is required for radiosensitization. Other investigators have produced results that are consistent with this suggestion[128].

It has been determined that resistance to radiation can result, at least in part, from an increase in the proficiency of repair of radiation-induced damage[139]. There is a strong correlation between radiation resistance and cisplatin resistance[112, 140], especially in head and neck tumors, which suggests that cisplatin resistant and radiation resistant cells share a common mechanism for resistance. In fact, some cisplatin-resistant cell lines have been shown to have an increased capacity for DNA repair[101-103]. Therefore, cisplatin may be expected to have a radiosensitizing effect in cisplatin resistant cells. This has proven to be true in several cell lines[141, 126, 90, 110, 107] and in some cases a greater radiosensitization was seen for the resistant cells compared to their parent cell lines.

The cisplatin resistant cells, SCC-25/CP, are also resistant to radiation (figures 4a and 4b). The SCC-25/CP cells were, therefore, examined for a response to combination cisplatin and radiation. Figures 34a and 34b show the drug normalized survival curves for 10 and 50 uM cisplatin treated cells, respectively, as a function of radiation dose. It was determined by the t values for radiation followed by cisplatin 60 minutes later, that synergy exists in the SCC-25/CP cell line at an LD 50of cisplatin (50 uM, figure 34b), over the entire radiation dose range. Perhaps the cisplatin-resistant cells are proficient in a type of repair, a type that is required for repairing radiation-induced damage and cisplatin is capable of inhibiting this repair. This could be different from the types of repair in the parent cell line that are required for the repair of radiation damage but are not inhibited by cisplatin. Therefore, the SCC-25/CP cell line should be examined to see what types of repair mechanisms are responsible for the resistance to cisplatin and cross-resistance to radiation. If a repair mechanism exists that is distinct from the repair in the parent cell line, then this type of repair could be important in achieving radiosensitization in other cell lines.

The cisplatin resistance of the SCC-25/CP cell line is caused by many factors. These factors include an altered uptake of the drug, a reduced level of cisplatin-DNA cross-linking, increased cytosolic binding and metabolism of cisplatin, and a 2-fold increase in sulfhydryl content[119]. It is possible that the toxic-scavenging ability of glutathiones plays a major role in the resistance of the cells to cisplatin. However, glutathione levels are limited and not inducible by cisplatin. Therefore, it is possible that the radiosensitization that occurs in the SCC-25/CP cell line, is due to radiation-induced damage, and an eventual exhaustion of glutathione scavengers (for a high enough drug

concentration) with a consequent redox stress, caused by cisplatin. This idea hypothesizes two separate targets for the combination interaction. This theory does not account for the additivity seen in the SCC-25 cell line. In fact, this theory might predict a greater degree of radiosensitization in the parent cell line.

The mechanism of interaction for cisplatin radiosensitization that has been observed in many systems and in the cisplatin-resistant model in this project, likely involves at least all three of the factors discussed: cell cycle effects, inhibition of radiation-induced damage and sulfhydryl-group scavenging. Other factors that could play a role in sensitization are signaling and gene expression, and stress and enzyme responses.

There are several studies, both experimental[127-129] and clinical[117, 118] that report only additive cell kill for various combinations of cisplatin and radiation. The results of this study are consistent with this finding for most of the combinations investigated. The results can also be interpreted in the context of a phenomenon similar to the adaptive response.

It is well known that small radiation doses can protect cells against subsequent radiation damage in a variety of cell types[120, 121]. This effect has been termed the adaptive response and it is known to depend on several factors. These factors include the doses of both the pretreatment and challenge radiation, and the time between pretreatment and challenge doses[125, 141]. There are some agents, other than radiation, that can produce a protective effect against subsequent radiation damage[125, 128, 148]. Therefore,

it is hypothesized that the type of agent given, or more specifically the type of damage caused by the agent given, is also a factor in the adaptive response.

Several investigators have observed that the survival curve for single doses of radiation has two distinct components at low doses for different cell types[133, 134, 142]. These radiation survival curves are typically characterized by a region between zero and 0.5 Gy that is hypersensitive to radiation (the HRS region). This is followed by a region of the curve beginning between 1 or 2 Gy that is radioresistant in comparison (the IRR region). The cisplatin survival curve determined in this study showed a region between zero and 1 uM that was slightly steeper than the rest of the curve (figures 1a and 1b). However, since this range consists of only two data points (0.1 and 1 uM) it would be premature to declare this region of the survival curve as hypersensitive. The biphasic behaviour of cisplatin survival has previously been noted in another cell line[72], so it is not inconceivable that it might exist for these cells as well. The radiation survival curve, shown in figures 2a and 2b, has no distinct HRS or IRR region. To determine the presence of HRS and IRR regions of cisplatin and radiation survival in this cell line, the survival would have to be characterized for several concentrations of cisplatin between zero and 5 uM and for several doses of radiation between zero and 2 Gy. An appropriate experimental method and assay would have to be used to reduce the error inherent in survival measurements.

It has recently been recognized that the range of challenge doses over which an adaptive response is normally seen, is comparable to the HRS region[133]. This has lead investigators to theorize that the adaptive response elicited by pretreatment is an induction of increased radioresistance (IRR) over the HRS region. It has also been observed that the induction of an adaptive response depends on the pretreatment dose[126, 121, 135, 125, 141]. This is evidence for the existence of a threshold for inducing a protective response.

In this study, treating with radiation 60 minutes before cisplatin resulted in a consistent increase in radiation survival for 0.1 uM. The increase was seen for 0.05 to 2.5 Gy (figure 18a). Some survival increase was also seen over the concentration range of 1 to 5 uM for doses of 0.05, 0.25 and 1.0 Gy (figures 18b and 18c). The existence of both HRS and IRR regions for cisplatin survival has been verified[72], and induction of the adaptive response by conditioning doses of radiation has been well substantiated[121, 129]. Therefore, it should not be surprising that radiation can induce a response.

Excision repair, and more specifically single strand breaks in DNA, have been implicated as a trigger for induced protection against subsequent damage[138, 139]. There is evidence that the induced response may involve increased production of improved enzymes, therefore, increasing DNA repair[122]. It is not known whether the induced response affects only the type of damage which caused the induction, or if several repair pathways are enhanced, and therefore DNA damage of one kind can induce protection

against a different type of DNA damage. It seems logical that the induced response would at least protect against subsequent damage of the same type. Both radiation and cisplatin are known to cause single strand breaks in DNA [19]. One might therefore expect small amounts of each to protect against challenge treatment by the other. This is consistent with the observation that cisplatin can protect against subsequent radiation, and that radiation can protect against subsequent cisplatin found in this study.

Since no radiation doses below 0.05 Gy were explored in this study, it can only be assumed that the cisplatin-protection induced by radiation is triggered by a threshold dose of radiation. The survival increase is not present for radiation doses above 1.0 Gy which may merely be the result of toxic cell kill outweighing the increased survival due to cisplatin-protection. It can be seen from figures 18a through 18e that the induced survival increases for increasing doses of radiation pretreatment. This occurs most prominently for 0.05 Gy, and is present to some extent for all radiation doses. This observation shows that the extent of cisplatin-protection that is induced depends on the magnitude of the radiation pretreatment.

Similarly, treating with radiation 10 minutes before cisplatin resulted in a radiation survival only for 0.1 uM of cisplatin. The increase was seen for radiation doses of 0.25, 1.0, 2.5 and 7.0 Gy (figure 21a). These observations provide support for the case of an inducible response that is triggered by a threshold dose of radiation. Perhaps radiation can illicit a protective response against any agent causing a certain type of

damage, produced by cisplatin in this case. The protective effect by radiation against cisplatin damage is noticeably lower relative to the corresponding 60 minute schedule. This is evidence that the protective effect is inducible and occurs on a time scale of at least one hour and possibly longer.

An alternative to radiation inducing a protective response against cisplatin damage is that cisplatin given after radiation treatment improves radiation survival. This situation is demonstrated in figures 12a to 12d. Treating with cisplatin 10 minutes after radiation resulted in a radiation survival increase over the dose range of 0.25 to 2.5 Gy. The increase was only seen for 0.1 uM of cisplatin. The radiation dose range over which the survival increase was observed is the same range over which hypersensitivity to single doses of radiation has been observed[133, 135]. This is also the dose range over which certain cell lines have demonstrated an adaptive response[125, 141]. However, in this experiment, it is the effect of cisplatin given *after* radiation, compared with single agent radiation, that is being investigated. Although a survival increase is seen for some cisplatin-radiation combinations, it is difficult to interpret these results as evidence that cisplatin can induce a radioprotective response *after* radiation damage has occurred. Of course, this could be an example of the importance of timing the window of response induction and the window of response effectiveness mentioned above. The time line of response for both radiation and cisplatin, in this case, would have to be quite complex and at least bimodal to account for the results in the previous combination schedules. An alternative, albeit simple, explanation is that radiation induces a response so that

subsequent cisplatin is less effective at cell kill. This conclusion is more obvious and seems more appropriate given that only survival curves are being used at this point to infer mechanistic effects.

Treating with cisplatin 60 minutes after radiation resulted in a radiation survival increase over the dose range of 0.05 to 1.0 Gy. The increase was most marked for concentrations of 0.1 and 20 uM cisplatin. Figures 15a to 15d show how the survival at each radiation treatment is affected by cisplatin. The conclusion for this experiment is the same as for the previous schedule with a 10 minute interval between radiation and cisplatin. The conclusion is that radiation induces a response that makes subsequent cisplatin exposure less effective at cell kill. Once more, the survival increase is slightly greater for cisplatin given 60 minutes after radiation than for cisplatin 10 minutes after radiation. This experiment also supports the idea that the longer the interval between the two treatments, the greater the extent of response induction, at least over the time intervals of 10 and 60 minutes used in this study.

Similarly, the case where cisplatin pretreatment is seen to affect radiation survival can be looked at as how the addition of radiation affects prior cisplatin treatment. Figures 22 and 23 for a 10 minute interval and 24 and 25 for a 60 minute interval show that no affect is achieved.

Treating with cisplatin 60 minutes before radiation resulted in a radiation survival increase over the dose range of 0.05 to 1.0 Gy. The increase was highest for 0.05 Gy and was seen for concentrations of cisplatin between 1 and 20 uM (figures 7a and 7b). The radiation dose range over which the survival increase was observed is the same range over which hypersensitivity to single doses of radiation has been observed[133, 135]. The HRS region has typically been observed for radiation doses of zero to 0.5 Gy. This is also the dose range over which certain cell lines have demonstrated an adaptive response[125, 141]. The ability of cisplatin to induce radioresistance over the HRS region of radiation survival has been observed[148, pers. comm. Skov, 1996]. Since the protection against radiation induced damage offered by cisplatin pretreatment occurs over the radiation dose range 0.05 to 0.25 Gy, these results are consistent with the hypothesis that the adaptive response elicited by pretreatment is an induction of increased radioresistance (IRR) over the HRS region.

The increase in survival is only seen for cisplatin concentrations greater than 1 uM, implying that a threshold exists for the induction of radioresistance. This is consistent with the findings in a number of other investigations[125, 141]. It can be seen from figures 7a through 7d that the induced radioresistance increases for increasing concentrations of cisplatin pretreatment. This occurs for the radiation dose range of 0.05 to 1.0 Gy, above which dose there is no survival increase. This observation shows that the extent of radioresistance induced depends on the magnitude of the cisplatin pretreatment.

Treating with cisplatin 10 minutes before radiation did not change the radiation survival (figures 8 and 9). It is hypothesized that the administration of cisplatin only 10 minutes prior to radiation does not trigger the mechanism of radioresistance at the time critical for radioresistance to be induced. In other words, the time line for induction of the radioprotective response may be the result of two components; a time window in which the response is induced by the pretreating agent, and a time window in which the response can be effective in protecting cells against the second agent. Therefore, in this case, cisplatin treatment 10 minutes prior to radiation may trigger an inducible response, but the response does not occur during a time critical for repair of the radiation-induced damage.

The induction of radioresistance has typically been observed 5 to 8 hours after pretreatment[125, 141, 127]. However, these observations are for x-ray pretreatment. The time line for the effect induced by cisplatin pretreatment may be different, especially if the radioresistance induction is based on the number of strand breaks or some other biological endpoint, for which there is evidence[139]. There is a report of induced radioresistance with the desmid *Closterium*, which occurred 1 hour after radiation pretreatment and peaked at 6 hours[150]. Other investigators have observed an adaptive response for cisplatin treatment 1 hour before radiation in mammalian cells that are hypoxic[148] and in mammalian cells that are not hypoxic[pers. comm. Skov, 1996]. It is not possible to define a duration or a peak time for the induced effect, as 1 hour was the longest interval investigated in this study. However, from the work reported here, it can

be concluded that the 10 minute interval between cisplatin pretreatment and radiation challenge is not sufficient to induce a protective response in SCC-25 cells. This supports the existence of a time dependence for the protective response in this cell line, and is similar to the result found for radiation pretreatment with cisplatin challenge.

It has been determined that continuous exposure to low concentrations of cisplatin results in an induced resistance to subsequent cisplatin treatment[125]. This suggests that cisplatin induced damage, perhaps single strand DNA breaks, can induce a protective response against subsequent DNA damaging agents. This is known to be true for cisplatin pretreatment and challenge doses of radiation, which further supports the idea that both the induction of the response and the effectiveness of the response are triggered by a type of damage caused by both cisplatin and radiation. This may also explain the observed correlation between radiation resistance and cisplatin resistance[112,140]. This cross resistance was also observed for the SCC-25/CP cell line used in this study (figures 4a and 4b). More specifically, if radiation resistance is at least in part caused by increased repair, which has been suggested[129,139] and is consistent with the observed correlation between intrinsic radioresistance and the extent of IRR[151], then cells proficient in radiation repair may also be proficient in cisplatin repair.

One would, therefore, expect a cisplatin resistant cell line treated with radiation to be less sensitive than the parent cell line, which is true for this study. Adding a conditioning concentration of cisplatin as combined therapy with radiation might not

necessarily improve survival, since the induced resistance, or IRR, may already be activated to its potential for resistant cells. If a concentration of cisplatin was used in a schedule that was known to inhibit the IRR response, then the effect of combined treatment with radiation may be to decrease survival relative to radiation alone. It was found in this study that adding 50 uM of cisplatin 60 minutes after radiation decreased the survival markedly relative to radiation alone (figure 34b). This effect occurred over the entire dose range tested (0.05 to 7.0 Gy) and was not see to the same extent with 10 uM cisplatin (figure 34a). The fact that over the radiation dose range from zero to 2.5 Gy, the SCC-25/CP cell survival is equivalent to the parent cell survival may imply the presence of a relatively sensitive region of the survival curve (figures 4a and 4b). The SCC-25 and SCC-25/CP survivals diverge for radiation doses greater than 2.5 Gy, which could be the beginning of an IRR region for SCC-25/CP cells. This is consistent with radiation doses of 0.05 to 0.5 Gy in the HRS region, and may extend to 2.5 Gy for this cell line. Cells may be hypersensitive to pretreatment doses of radiation in this region, so subsequent cisplatin exposure causes more than additive cell kill (figure 34b). However, one might expect the radiation pretreatment to result in a protective effect for radiation doses beyond the presumed HRS region. Radiation doses greater than 2.5 Gy do not interact with cisplatin to produce a protective effect.

The results for the SCC-25/CP work are not detailed enough to infer much about the induction of a protective response. It is clear that further studies are required to

define the potential HRS and IRR regions for the SCC-25/CP radiation survival curve and to test for the presence of an adaptive response.

Conclusions

Combination treatment with cisplatin and radiation generally resulted in additive cell kill in the SCC-25 cell line when cisplatin was administered before radiation in the treatment schedule. This was observed for a 10 minute time interval between the two treatments as well as for a 60 minute time interval. Also seen for the 60 minute time interval was a tendency towards antagonism for cisplatin concentrations with very low doses of radiation (0.05 Gy). Antagonism between cisplatin and radiation was considerably more prominent when radiation was administered first in the treatment schedule. The observed antagonism occurred consistently for combinations with low radiation doses (less than 1.0 Gy) across a range of cisplatin concentrations. These results are not consistent with previous reports of cisplatin's radiosensitizing ability. It is difficult to define the role of cell cycle kinetics and repair inhibition in the observed cisplatin-radiation interaction until these processes are investigated in detail for the SCC-25 cell line.

The antagonism can be better explained in the context of the adaptive response. The results from this study are consistent with previous observations that cisplatin can induce radioresistance when administered one hour or more before radiation treatment. The ability of radiation to induce resistance to subsequent cisplatin treatment, as observed in this study, has not previously been reported. However, there is some suggestion that a protective response may be elicited by a molecular trigger that can be activated by a

number of agents. The observed radiation-induced cisplatin protection in this study is consistent with this theory.

Combination cisplatin-radiation treatment was observed to interact synergystically in the SCC-25/CP cell line when radiation preceded cisplatin by 60 minutes. To determine whether or not this provides support for the suggestion that cisplatin preferentially radiosensitizes repair proficient cells, the repair mechanisms involved in cisplatin and radiation resistance in the SCC-25/CP cell line should be investigated. In particular, the activity of the repair mechanisms over the entire radiation dose range should be examined in an attempt to elucidate a connection between repair activity, the HRS region of the survival curve, and the potential for a synergistic interaction between cisplatin and radiation.

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