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TUMOR CELL KILLING BY INCORPORATED RADIOISOTOPES

by

WILSON HUNG-YAN ROA

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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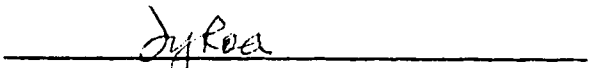

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DEDICATION

To Elim, my financee, who shares the grace of life with me.

ABSTRACT

The efficiency of tumor cell killing by radioisotopes bound intracellularly through sensitizer adducts was studied *in vitro*.

Nitroaromatic radiosensitizers become metabolically bound selectively to hypoxic cells and over 10^9 adducts/cell can be tolerated as non-toxic. Mouse fibrosarcoma cells (EMT-6) were incubated in hypoxia in the presence of ^{3}H-misonidazole or ^{125}I-azomycin riboside for up to 4 hours and the amount of metabolically bound ^{3}H or ^{125}I per cell determined. Cells were stored as monolayers at 22°C, in suspension culture at 4°C and in 8% DMSO at -196°C for various times to facilitate the accumulation of radioactive decays. Cells were then plated for colony-forming assays at 37°C.

At 22°C in monolayers, EMT-6 cells tolerated at least 950 ^{3}H- and 1700 ^{125}I- decays/cell as non-toxic. This suggests that in excess of 10^6 ^{3}H and 10^5 ^{125}I isotopes per cell would be required to observe any radiation inactivation. At 37°C where DNA repair is most efficient, even greater numbers of incorporated isotopes would be required to produce cell killing. Significant cell killing was observed for this level of isotope decay in cells held at 4°C, where little or no repair occurs. If cells were frozen in the presence of 8% DMSO to accumulate isotope decays, they were more resistant to radioactive inactivation than cells in liquid at 4°C. Presumably this was due to the elimination of most indirect effects of radiation.

These studies indicate that the investigated radioisotopes bound to hypoxic cells in tumors through sensitizer adducts are unlikely to produce sufficient cell kill to be useful as an adjunctive tumor

treatment. Cellular repair mechanisms at 37°C are expected to efficiently remove most if not all potentially lethal and/or sublethal damage induced in mammalian cells at the low dose-rates expected from these levels of isotope decay. The radiation effectiveness of incorporated isotopes on tumor-specific antibodies might be similar to the effectiveness of these labelled adducts.

Protection of EMT-6 cells against external beam ionizing radiation was demonstrated with dimethyl sulphoxide, hypoxia, dithiothreitol and freezing. The dose reduction factor for dimethyl sulfoxide was increased by the addition of either hypoxia or dithiothreitol. For cells in 8% DMSO in frozen state (-196°C), however, the addition of other chemical protectors did not add further protection to what had been achieved by the low temperature alone. These chemical modifiers appear to act by either scavenging diffusible free radicals or by chemically repairing target damage induced by diffusible free radicals. The lower killing efficiencies of incorporated isotopes in cells in frozen state compared to cells held at 4°C can be attributed to the immobilization and decay of potentially lethal, diffusible free radicals.

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CHAPTER 1

INTRODUCTION

Gray et al. (1) were first to suggest that the oxygen concentration in tumor cells at the time of radiotherapy might influence the curability of some human cancers. Subsequent evidence supporting this premise came both from basic laboratory research and clinical investigations. To demonstrate that hypoxic cells are a factor in the radiocurability of a tumor, tumors were made artificially hypoxic by blocking the blood supply (2) or killing animals by asphyxiation (3) prior to irradiation. The different surviving fractions indicated the degree and importance of hypoxia in radiation resistance. Further evidence of hypoxia in both human and animal tumor models has come from measurements of oxygen concentration within tumors using oxygen electrodes (4,5). Hypoxic cells are known to be about 2.5 to 3.0 times more resistant to ionizing radiation than are oxygenated cells (30). Tannock (6,7) indirectly demonstrated the presence of hypoxia in tumors by identifying proliferating cells which had incorporated ^{3}H-thymidine and the necrotic core which had not. Intermediate to these two areas was a nonproliferating zone and presumably was low in oxygen content which consistently had a lower labelling index. By using high pressure oxygen during radiotherapy, clinical investigators showed an enhanced cure rate which they attributed to elevated oxygen tensions in the tumors (8-11). Another clinical study showed that low hemoglobin levels correlated with high recurrence rates after radiotherapy in carcinoma

of the cervix (12). Hemoglobin levels elevated by transfusions before radiotherapy to over 12 g percent to alleviate anemia resulted in an improved radiocure rate (12) which could have been indicative of improved tumor oxygenation.

Results from aforementioned and other experiments have strongly suggested that hypoxic cells are present in tumors and that their presence affects the eradication of tumors by ionizing radiation and some chemotherapeutic agents. Proposed techniques for overcoming this treatment resistance include the use of hyperbaric oxygen therapy (13,14), fractionated irradiation schedules to maximize reoxygenation (15) and high-LET radiations (16). Other adjuncts to radiotherapy include hypoxic cell sensitizers and agents toxic to hypoxic cells such as hyperthermia and bioreductive alkylating agents (17,18). Some of these techniques are currently undergoing evaluation in clinical trials.

Previous research with hypoxic cell radiosensitizers had shown that nitroaromatic drugs become metabolically bound to the molecules of hypoxic cells, selectively (19). Sensitizer adduct formation has been developed as a method for identifying hypoxic cells in multicellular spheroids *in vitro* and in human cancers (20,21). Studies by Garrecht and Chapman (22) were designed to determine if hypoxic tissues in animals selectively bind ^{14}C-Misonidazole (^{14}C-MISO). After multiple drug injections it was determined using both autoradiographic and scintillation procedures that the level of ^{14}C retained in the tumor was 2-15 times higher than that retained in all other normal tissues except liver (22). These studies gave further evidence that hypoxic cell sensitizers tagged with an appropriate label that could be detected

by histological or noninvasive techniques could become a clinical marker for hypoxic tumor cells.

Biophysical characterizations of the hypoxic cell reduction mechanisms have shown that greater than 10^9 MISO adducts can be tolerated by both EMT-6 (mouse fibrosarcoma) and Chinese hamster V79 cells without evidence of cytotoxicity (23). MISO has become the standard (24) against which comparisons of radiosensitizing activity and sensitizer adduct measurements should be made. Another nitroimidazole, azomycin riboside (AZR), first synthesized by Rousseau et al. (25), was found to have both radiosensitizing and cytotoxic properties similar to that of MISO (26,27). Its octanol/water partition coefficient, however, is similar to that of the investigational clinical sensitizer SR-2508. Hence like SR-2508, AZR should have a lower potential to induce neurotoxicity than misonidazole due to its low lipophilicity (28,29). Both MISO and AZR were available for this study.

The development of a histological or non-invasive technique for routinely measuring hypoxic cells in human tumors could have a major impact on treatment strategies in clinical oncology. The metabolically bound sensitizers are capable of exhibiting a high degree of selective concentration within the hypoxic (treatment resistant) tumor cells. Moreover, the covalent nature of sensitizer binding allows for a steady and prolonged retention within viable cells sequestered in hypoxic tumor zones. This study was designed to determine if such radiosensitizer adducts could be used as selective carriers of radioisotopes to the treatment resistant hypoxic cells within tumors and effect cell inactivation by local radiation ("suicide") effects. Tumor cell killing

by incorporated radioisotopes will be most effective if the particles emitted have ranges similar to the cell dimension. Otherwise ionizing potential will be delivered to great distances away from hypoxic cells and therefore dilute the radiation effect. In this study a β^- emitter, tritium (^3H) and an auger electron emitter, iodine 125 (^{125}I) were available on MISO and AZR, respectively. By using high specific activity compounds, we proposed that appreciable in situ radiotherapeutic effect might be exerted upon treatment resistant, hypoxic cells. This cell "suicide" technique could serve as an adjunct to conventional radio- and chemotherapy procedures which are effective against oxygenated tumor cells.

The proposed technique has similarities to the proposed use of radioactively-labelled poly- and monoclonal antibodies to tumor-specific antigens to effect cell inactivation. Radioactively-labelled antibodies are expected to selectively associate with both oxygenated and hypoxic cells in tumors and consequently may not require the action of an additional treatment modality. On the other hand, the maximum number of tumor specific antibodies expected to associate with any one tumor cell is in the order of 10^5 (36). The fact that over 10^9 sensitizer adducts can be tolerated by tumor cells in culture warrants an evaluation of this technique for delivery of short-lived, short-range radioisotopes for hypoxic tumor cell suicide.

So called suicide experiments were first designed by Hershey and his associates in 1951 (31). The experiments, in principle, were carried out as follows. Cells were cultured with a radioactive precursor to label a specific type of molecule with radioactive atoms. The labelled

cells were either frozen or kept at low temperature, so that all metabolic processes ceased and the only event occurring in cells was the decay of incorporated isotopes which effected a radiation exposure. After allowing for a specific number of decays, the cells were brought back to 37°C and the biological effects of decay of the radioactive atoms were measured.

Mouse leukemia (L5178Y) cells were labelled with one of four ^{3}H-labelled precursors (32), namely ^{3}H-thymidine, pulsed ^{3}H-uridine, pulsed ^{3}H-uridine plus cold chase or ^{3}H-lysine. It was found that the cells labelled with ^{3}H-thymidine for one generation were killed three times more effectively than cells pulse-labelled with ^{3}H-uridine. Similarly, cells labelled with ^{3}H-thymidine were killed five times more efficiently than those pulse-labelled with ^{3}H-uridine followed by a cold chase for one generation, and eight times more efficiently than those labelled with ^{3}H-lysine for 30 min. This result suggested that the effectiveness of killing by ^{3}H decay depends upon the location of the radioactivity within cells and follows the following sequence of most sensitive to least sensitive sites: DNA > mRNA > rRNA > tRNA > amino acids. In ^{3}H-thymidine labelled mouse leukemia (L5178Y) cells, the survival curve of unifilarly labelled cells has a D_o value of 167 decays/cell, while the survival curve of bifilarly labelled cells has a D_o value of 80 decays/cell (32).

Kassis et al. (33) examined extensively the radiotoxicity of iodine-125. They custom-made ^{125}I labelled compounds targeted to specific cell components. These included ^{125}I-deoxyuridine (^{125}I-dU) specific for DNA incorporation, ^{125}I-dihydrorhodamine (^{125}I-DR) specific for

mitochondria incorporation (∿ 96% localized in the cytoplasm) and $^{125}I^-$ for extracellular labelling. V79 cells were suspended in various concentrations of the radionuclides and incubated for 18 hours. Instead of accumulating the decays in cells at a lower temperature like the classical suicide experiments, cells were plated for survival assays after the completion of the isotope binding procedure.

D_o value generated from the survival curve of V79 cells into which ^{125}I-DR had been incorporated is 109 mBq per cell. The total number of disintegrations in the target cell was estimated from the area under the cellular uptake versus time curve to be 5580 (1.55 Bq/hr) (33). Assuming the same rate of decay for the iodine-125 incorporated in DNA, the survival curve for the ^{125}I-dU incorporated cells has a D_o value of ∿ 81 decays per cell (1.3 mBq/cell). The killing effect of extracellular iodine-125 was negligible. It is concluded that the cytotoxic effect of iodine-125 is primarily dependent on its proximity to nuclear DNA. Iodine-125 concentrated by cells but not bound to or incorporated into DNA produced low-LET-type survival curves, whether localized in the cytoplasm or more diffusely distributed within the cell. ^{125}I fixed to the plasma membrane (34) or free in medium (35) did not cause significant cell death.

CHAPTER 2

DOSIMETRY OF INCORPORATED ISOTOPES

Tritium is one of the heavy and unstable isotopes of hydrogen. It has an atomic weight of 3 and decays to form helium, together with the emission of a β-particle. It emits no γ-ray. Tritium has been widely used in biology research because of its ability to produce autoradiographs of very high resolution. Numerous metabolically active substances have been labelled with tritium and their fates in vitro have been studied extensively. As many of these substances are produced with a very high specific activity, it is possible to reach levels of tritium incorporation that may bring about changes in the normal metabolic processes by intracellular irradiation. The biological effects of tritium are related to the β-irradiation (43). Figure 1 shows the energy and the relative frequency of tritium β-rays (44). The maximum β-ray energy from ^{3}H decay is 18 KeV and the mean energy of 5.6 KeV is the lowest found among the radioactive elements. The path length of the mean energy β-ray from tritium in unit density is approximately 1 μm, and the distance that a maximum energy β-ray from tritium can travel is 6 μm (45). The paths of most low energy β-rays are very tortuous and the average range from the decay point of a tritium atom is probably less than the total distances traveled.

Radioactive ^{125}I decays by electron capture (t 1/2 = 60 days) to an excited state of ^{125}Te which undergoes immediate decay by internal conversion (93%) or by a 35-KeV γ-ray emission (7%) to the ^{125}Te daughter

FIGURE NOT SHOWN
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Figure 1: β-Spectrum of Tritium (Robertson & Hughes, 1959)

ground state. Due to inner shell vacancies produced, a complex series of electron shell rearrangements occur, resulting in the emission of low-energy X-rays (3.8 - 31 KeV) and a cascade of low energy electrons (36). The dose distributions resulting from the photon emission is relatively constant and contributes very little dose to the cell in which the decay occurs. An average of 7.5 KeV photon energy was obtained per ^{125}I disintegration in thyroid tissue (46). The complex cascade of low energy electrons are produced predominately by the nonradiative Auger, Coster-Kronig and super Coster-Kronig processes as illustrated in Figure 2 (38). The progressive transitions of energy result in a dense shower of low energy electrons having a maximum range of 0.02 μm in unit density (39). The mean range of such electrons is still not precisely known today. These low energy Auger electrons have been shown to cause specific subcellular damage when used to radiolabel nucleotide precursors (34,34). The relevant physical data for both ^{3}H and ^{125}I are listed in Table 1.

In order to work out the dosimetry of the incorporated radioisotopes, the average dimensions of the EMT-6 cells used in this study were as follows:

The mean cell volume of asynchronously growing EMT-6 cells in suspension is 1690 μm^3 (40).

The radius (r) of EMT-6 cell can be calculated by using

$$4/3 \pi r^3 = 1690 \mu m^3$$

Hence $r = 7.39 \mu m$

Assuming the ratio of cell radius to nucleus radius as 1.7 (41),

FIGURE NOT SHOWN
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Figure 2: Nonradiative Atomic Vacancy Transitions of Low Energy Electrons in Iodine-125 (Sastry & Ras, 1984)

TABLE 1

PHYSICAL DATA OF ^{3}H AND ^{125}I ISOTOPES

ISOTOPE	PARTICLES EMITTED ON DECAY	HALF LIFE	MAX. ENERGY OF PARTICLE	MEAN ENERGY OF PARTICLE	MAX RANGE OF PARTICLE IN TISSUE	MEAN RANGE OF PARTICLE IN TISSUE
^{3}H	β^-	12.26 yr	18 KeV (37)	5.7 KeV (37)	6.0 μm (45)	1 μm (48)
^{125}I	Auger Electron, Coster-Kronig Electron and Super Coster-Kronig Electron	60 day	500 eV (39)	Not precisely known (39) but less than 500 eV	0.02 μm (39)	Not precisely known (39)

the radius of EMT-6 cell nucleus can be calculated as

$$\frac{7.39\ \mu m}{1.7} = 4.4\ \mu m$$

Although the cytoplasm of EMT-6 cells changes in gross morphology when the cells are grown as monolayers, the volume of the nucleus will likely remain similar (41).

Assuming 1) a uniform distribution of radioisotope incorporated throughout the cells and 2) that only the decay occurring within the nucleus are effective in producing cell kill, the effective decays per cell can be calculated as

$$\text{Decays per cell} \times \frac{\text{mean vol. of nucleus}}{\text{vol. of EMT-6 cell}}$$

Hence the effective decays per cell for the ^{3}H-MISO study

$$= \text{decays per cell} \times \frac{4/3\pi \times (4.4\ \mu m)^3}{1690\ \mu m^3}$$

$$= 0.21 \times \text{decays per cell}$$

The assumption of a uniform isotope incorporation intracellularly correlates well with the experimental data. The assumption predicts the percentage of MISO incorporated in the nucleus as

$$\frac{4/3\ \pi\ (4.4\ \mu m)^3}{1690\ \mu m^3} \times 100\%$$

$$= 21.1\%$$

The experimental value using ^{14}C-MISO is 18.5% (Table 2).

The assumptions applied to the ^{3}H-MISO study is not suitable for the ^{125}I-AZR study. Realistically, Auger electrons released at locations more than 0.02 μm from the DNA molecule (critical target) will not

TABLE 2

INTRACELLULAR DISTRIBUTION OF BOUND ^{14}C-MISO*

	% UPTAKE BY NUCLEI	% UPTAKE BY CYTOPLASM
Exp. 1	22.45%	77.55%
Exp. 2	14.42%	85.58%
Exp. 3	15.49%	84.51%
Exp. 4	21.58%	78.42%
Mean	18.49 ± 4.11	81.52 ± 4.11

*Unpublished data from J. Lee and J.D. Chapman

contribute to the cell kill because of the short range of the electrons. It has been shown that only about 1% of MISO bound to cells is bound to the deoxyribose nucleoprotein (23). If one assumes a similar intracellular distribution for AZR, the fraction of ^{125}I that actually binds directly to DNA will be small. This implies that the dose delivered to the genetic target by the intracellular ^{125}I-AZR can be 20-fold smaller than that by the ^{3}H-MISO. However, Auger electrons have a relatively high LET value $\sim$ 15 to 25 KeV/μm (38) and will be more efficient than the β-particle of ^{3}H in cell kill.

Further, characteristic x-rays and the gamma-ray liberated by the ^{125}I decays might affect the neighbouring non-hypoxic cells. In order to estimate this dose, assumptions will need to be made. Calculations are done as follows:

Consider a 1 mm diameter sphere of hypoxic cells.

Number of cells in this spherical volume

$$= \frac{\text{volume of 1 mm}^3\text{ sphere}}{\text{mean volume of a cell}}$$

$$= \frac{4/3\ \pi\ (0.5\ \text{mm})^3}{4 \times 10^{-6}\ \text{mm}^3}$$

$$= 1.3 \times 10^{5}\ \text{cells}$$

If each cell accumulates a total decay of 1700 (see later chapters) and each decay liberates an average of 7.5 KeV photon energy (46), the total photon energy released from the hypoxic sphere

$$= 1.3 \times 10^{5} \times 7.5 \times 1000 \times 1.6 \times 10^{-19}\ \text{J}$$

$$= 1.56 \times 10^{-10}\ \text{J}$$

Now suppose we are interested in the dose absorbed in the circumferential non-hypoxic cells with a radius of 1 half value thickness

(HVT) with the HVT for 7.5 KeV phone $\sim$ 0.2 mm Al(62).

HVT of tissue equivalent hence

$$= \text{HVT of Al} \times \frac{\text{density of Al}}{\text{density of tissue}}$$

$$= 0.2 \text{ mm} \times \frac{2699}{1000}$$

$$= 0.54 \text{ mm}$$

This is approximately equivalent to a spherical volume of $4/3\ \pi\ (0.54\ \text{mm})^3$, i.e. $0.66\ \text{mm}^3$ or a mass of 6.6×10^{-7} kg.

Finally, taking the labelled hypoxic 1 mm diameter sphere as a point source, dose delivered to a HVT of the circumferential non-hypoxic cells

$$= \frac{1.56 \times 10^{-10}\ \text{J}}{6.6 \times 10^{-7}\ \text{kg}}$$

$$= 2.4 \times 10^{-4}\ \text{Gy}$$

This low dose is relatively insignificant in its contribution to tumor cell kill.

CHAPTER 3

MATERIALS AND METHODS

CELL CULTURE

EMT-6 fibrosarcoma mouse tumor cells were cultured as monolayers in 125 cm^2 tissue culture flasks (Corning). Confluent cells were trypsinized and transferred twice weekly in standard Minimal Essential Medium (Gibco) or Waymouth's Medium containing 5% fetal calf serum (Gibco). Approximately 5 x 10^5 cells were added to each flask for cell transfer. All cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

LABELLED DRUGS & RADIOPROTECTORS

Misonidazole (MISO) (1-(2-nitro-1-imidazole)-3-methoxy-2-propanol) was labelled with tritium at the C-2 position in July, 1985 and kindly provided for this study by Dr. J.A. Raleigh. The drug had a specific activity of 275 µCi/mg corrected for a radiochemical purity of $\geq$ 92% by TLC. The original concentration was 0.111 moles/l in absolute ethanol. Further dilutions were made using double distilled water for individual experiments.

^{125}I-Azomycin riboside (^{125}I-AZR) (^{125}I-1-(5'-iodo-5'-deoxyribofuranosyl)-2-nitroimidazole) was generously provided by Dr. L.I. Wiebe from the Faculty of Pharmacy, University of Alberta. The compound was prepared from AZR by an exchange labelling technique using ^{125}I-NaI in ethanol (47). The compound was purified by HPLC and had a chemical

purity of 99%. Its specific activity was 297 μCi/mg.

Both misonidazole and azomycin riboside contain the 2-nitroimidazole moiety. They are thought to be metabolically reduced within mammalian cells to the radical anion, nitroso, hydroxyl amine and amine derivatives by 1, 2, 4 and 6 electrons, respectively. The 2 and/or 4 electron reduction intermediates of 2-nitroimidazoles have been postulated as the reactive chemicals involved in the adduct-forming processes (63). Such adduct formations can be modified with intracellular oxygen and glutathione.

Dimethyl sulfoxide (DMSO) functions both as a radical scavenger and a cryoprotective agent. Absolute DMSO (Sigma) was diluted to 8% by volume in minimal essential medium (Gibco) and its radiation protection effectiveness was measured at room temperature, 4°C, -30°C and -196°C. Given the knowledge that DMSO [$(CH_3)_2SO$] has a molecular weight of 78.13 and a density of 1.103 g/ml at room temperature, the molar concentration of the 8% volume DMSO can be calculated as follows:

1) change weight/volume to mole/volume

$$\frac{\text{density x \% dilution}}{\text{M.W.}} = \frac{1.103\ \text{g/ml}\ (0.08)}{78.13}$$

$$= 1.13 \times 10^{-3}\ \text{mole/cc}$$

2) Change units to molar (M)

$$1.13 \times 10^{-3}\ \text{mole/cc} \times 1000\ \text{cc}$$

$$= 1.13\ \text{M}$$

Dithiothreitol (DTT) is a sulfhydryl compound. It can change the cytotoxicity of agents that are dependent on the redox cycle (48). DTT (Kodak) has a chemical formula of $HSCH_2(CHOH)_2OH_2SH$ with two SH

groups for each molecule. In this study, DTT was dissolved in minimal essential medium (Gibco) to yield a final concentration of 20 mM in which the cells were irradiated.

Hypoxia was produced by stirring the EMT-6 cell suspension for 1 hour in a hypoxic glass chamber (19,23). The cells were then transferred to cryotubes, sealed and shaken continuously for another hour at 37°C so any residual oxygen would be removed by cell metabolism. The cryotubes were 1.8 cc in volume. Each tube was filled with 1 cc cell suspension at a concentration of 10^6 cells per ml. The cells were irradiated in the oxygen-deficient environment of the cryotubes before plating for colony assays.

TECHNIQUE FOR HYPOXIC BINDING

EMT-6 cells at a concentration of 5×10^6 cells/ml were transferred to glass chambers designed to permit control of both temperature of the liquid culture and oxygen levels in the gas phase. These chambers were originally designed for radiobiological studies with suspensions of mammalian cells (19,23). The cell suspensions were gassed with humidified 5% CO_2 plus air for aerobic conditions and 5% CO_2 plus 95% ultra pure nitrogen (oxygen content < 5 ppm) for hypoxic conditions. Radiobiological hypoxia was achieved in cell suspensions stirred slowly in these chambers after 30 min of degassing with nitrogen mixtures at 1 l/min, and consequently a nitrogen gas flow rate of 1 l/min was maintained throughout the experiments. The temperature of the cell suspension was maintained at 37°C by suspending the chambers in a heated water bath.

Following a 1 hour degassing period, ^{3}H-MISO was added to the

chambers to achieve a final drug concentration of 100 μM, 500 μM, 1 mM and 2mM. ^{125}I-AZR was used at concentrations of 100 μM and 200 μM in a similar fashion.

DETERMINATION OF BOUND ISOTOPES/CELL

At various times following drug addition 0.1 ml samples of the cell suspension were removed through the sampling port in the chamber and added to 2.0 ml of PBS to preserve cell integrity. Samples were maintained on ice for less than 30 minutes and filtered through Whatman Glass Microfibre filters (Fisher Scientific) using a Millipore filtration manifold. Filters were washed twice with cold PBS before they were put into glass scintillation vials (Fisher Scientific) and dried for 2-3 hours in an oven maintained at 65°C. For the ^{3}H-MISO samples, 10 ml of scintillation fluid (Scintiverse I, Fisher Scientific) was added to each vial and the ^{3}H activity was determined using a Beckman LS7000 Liquid Scintillation Counter (Program 5). For the ^{125}I-AZR samples, direct counting of γ-rays was performed with a Beckman γ-counter at the Faculty of Pharmacy, University of Alberta.

CALCULATION OF RADIOACTIVITY/CELL

The decays of incorporated radioisotopes per cell were calculated using the following method:

1) Subtract background cpm (control samples).

2) Convert cpm (count per minute) to dpm (decay per minute)

$$\text{dpm} = \frac{\text{cpm}}{\text{counting efficiency}}$$

The counting efficiency was 40% for tritium and 100% for iodine-125.

3) As the experimental time was relatively short in comparison with the half-lives of the radioisotopes used and no significant loss of the covalently bound sensitizer adducts was detected for cells held at 22°C and 4°C (radioactivity assays indicated that more than 90% of the intracellular radioactivity was retained during and after the decay accumulation), the total decays accumulated in a cell were hence estimated as

$$\text{Total decays/cell} = \frac{\text{accumulation dpm per sample at end of binding} \times \text{time of storage}}{\text{number of cells in sample during binding}}$$

The molecular adducts of radiosensitizer incorporated per cell were also calculated as follows:

1) Convert dpm to μCi

$$\mu\text{Ci} = \frac{\text{dpm}}{2.2 \times 10^6 \text{ dpm}/\mu\text{Ci}}$$

2) Convert μCi to mg

$$\text{mg} = \frac{\mu\text{Ci}}{\text{specific activity of drug}}$$

(i.e. 275 μCi/mg for ^{3}H-MISO)

3) Convert mg to moles

$$\text{moles} = \frac{\text{mg (MW MISO = 201)}}{\text{molecular weight of drug}}$$

$$\text{therefore, moles} = \frac{\text{mg}}{201}$$

4) Convert moles to picomoles, 1 picomole = 10^{-12} moles.

TECHNIQUE FOR STORING CELLS TO ALLOW ACCUMULATION OF DECAYS

EMT-6 cells with incorporated radioisotopes were washed twice by centrifuging in 10 ml of HBSS (Gibco) at 1000 rpm for 10 minutes. The cells were then resuspended in spinner minimal essential medium (Mg^{2+} and Ca^{2+} free) for further treatments. Storage temperature was chosen as 22°C, 4°C and -196°C. For storage at 22°C, EMT-6 cells were plated in 100 x 10 mm petri dish (Falcon) in 12 mls of Waymouth medium (Gibco) and stored in a CO_2 gassed incubator. For storage at 4°C, cells were left in suspension inside a refrigerator adjusted to the temperature. For storage at -196°C, EMT-6 cells were suspended in the cryoprotective agent, dimethylsulfoxide (DMSO), at a final concentration of 8% by volume. The cell suspension was adjusted to 10^6 cells/ml and 1 ml was transferred to each cryotube (Nunc, 1.8 ml). After tightening of the caps manually, the cryotubes were sealed in capsules of cryoflex (Nunc) to prevent leakage. Cryotubes were then put into the vapour phase of a liquid N_2 tank by means of a specially designed cryotube holder (Figure 3) for 24 hours before they were strapped onto aluminum canes and plunged into liquid nitrogen (-196°C).

FREEZING TECHNIQUE AT -30°C AND THAWING

The low temperature of -30°C was achieved by adding dry ice to 95% ethanol in a thermos. The temperature was continuously monitored by a thermometer. This temperature was an approximation only as it was difficult to maintain temperature stability during transportation and irradiation. The cells were pretreated with 8% vol. DMSO as those samples cooled to -196°C.

After various times, the frozen vials were removed from the liquid

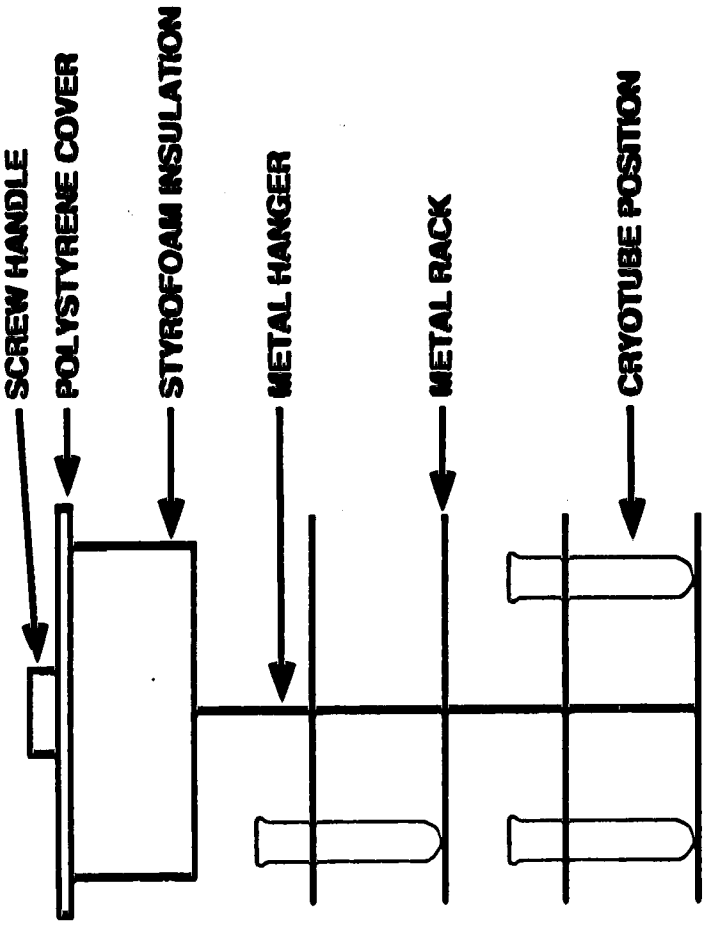

Figure 3: Picture of Cryotube Holder in N_2 Vapour

nitrogen and thawed by immersion in a water bath maintained at 37°C. The water was constantly stirred to ensure a uniform temperature in the bath. When all crystal residues disappeared, the cells in the suspensions were retrieved for further plating procedures as described below.

ASSAY OF SURVIVAL

Cell suspensions irradiated under various experimental conditions (by incorporated radioisotopes or external beams) were counted using a Coulter counter and a known number of cells were plated in 4 mls of Waymouth medium (Gibco) with 5% FCS and 1% erythromycin into tissue culture petri dishes. After an incubation period of 7 days, macroscopic colonies derived from single, surviving cells were rendered visible by fixation with 70% alcohol and staining with methylene blue. These numbers of colonies were divided by the number of cells originally plated to give the plating efficiency (P.E.) for the test condition. The ratio of this P.E.to the P.E. of the control unirradiated cells yielded the survival fraction (S.F.). Results were then plotted on semilogarithmic plot to facilitate interpretation.

DOSIMETRY OF GAMMA CELL

Fricke (ferrous sulphate) dosimeter was used to calibrate the gamma cell in the W.W. Cross Cancer Institute of Edmonton, Alberta. The basic reagents of the dosimeter were similar to the one proposed by Fricke and Morse (49). They were, namely, 2 g of $FeSO_4 \cdot 7\ H_2O$, 0.3 g of NaCl, 110 cc concentrated (95-98%) H_2SO_4 (analytical reagent grade) in water to make up a final volume of 5 liters of solution.

Ferrous ion has an absorption maximum at approximately 305 nm. It is therefore possible to determine the amount of iron oxidized ($Fe^{2+} \rightarrow Fe^{3+}$) due to the radiation imposed by using the non-irradiated sample as control and reading the optical density of the irradiated sample with respect to it. The volumes of the samples measured were 2 ml or greater. For the best results, the temperature of the sample in the spectrophotometer (Beckman) was noted, since the extinction coefficient has a large temperature coefficient of 0.7%/degree centigrade (50).

Given molar concentration of Fe^{3+}

$$= \frac{\text{O.D. (sample)} - \text{O.D. (Blank)}}{E\,[1 + 0.007\,(T-25)]}$$

The number of Fe^{3+} formed can be calculated as

$$\frac{\text{O.D (sample)} - \text{O.D. (Blank)} \times N}{E\,[1 + 0.007\,(T-25)]\,P}$$

where N = Avogadro's number

$= 6.023 \times 10^{25}$ molecules/mole

T = ambient temperature °C

E = molar extinction coefficient

= 2174 litres/mole - cm

$P = 1.016 \times 10^{3}$ gm/l

Now 1 cGy = 100 ergs/gm = 6.24×10^{13} eV/gm

and G = 15.6 molecules/100 eV

$$\text{Hence dose (cGy)} = \frac{\dfrac{\text{O.D. (sample)} - \text{O.D. (blank)} \times N \times \frac{100}{15.6}}{G\,[1 + 0.007\,(T-25)]P}}{6.24 \times 10^{13}}$$

Calibration of radiation doses vs exposure time for the gamma cell was plotted in Figure 4. The non-zero value of dose at time zero results from the radiation exposure to the samples during their

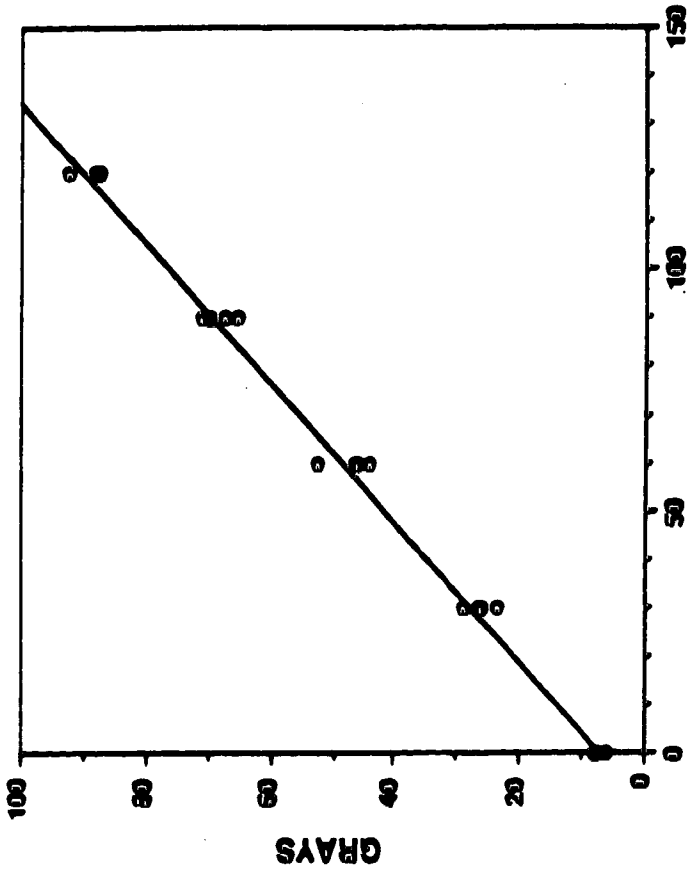

Figure 4: Calibration of Gamma Cell - January, 89

mechanical transportation to and from the irradiation position within the γ-cell. The dose accumulated by this up/down motion is a constant for each of the timed exposure. Positions of the Fricke's solution during calibration are fixed. A styroform holder with carved-out holes at equal distances from the centre was placed in a constant position at the base of the circular irradiation chamber. Radiation exposure to the separate samples should be identical in a same period of time. Although the ^{60}Co source decays and hence decreases in its radiation intensity over time, the duration of its use for the part of this study (3 months) is relatively short when compared with the half-life of ^{60}Co (5.26 yr). The calibration curve (Figure 4) was assumed to be appropriate for all the studies reported in this thesis.

CHAPTER 4

CELL KILLING BY INCORPORATED ISOTOPES

Tissue cultures provide convenient material for quantifying the relative efficiencies of different qualities and time patterns of radiation in producing cell death. This study involved measurements of radioactively-labelled sensitizer adducts *in vitro* and the storage of such labelled cells as monolayers at room temperature or as cell suspensions or in frozen matrix at low temperatures. Cell killing resulting from isotope decays was assayed by colony forming assays.

Following an accumulation of radioisotope decays for up to 4 days at room temperature (22°C), EMT-6 cells were plated for survival assays. ^{3}H-MISO was bound at a level which would produce 10 decays/hour/cell on average. EMT-6 cell killing by incorporated tritiated MISO was not different from the EMT-6 cell toxicity imposed by the cold MISO drug alone at the same concentration (Figure 5). Standard errors in the figures were calculated by the formula SF x $(\frac{\sqrt{N}}{N} \times 100\%)$ where SF is the survival fraction and N is the average number of formed colonies per plate. The difference of survival fractions between the hot and cold MISO was plotted against decays per cell in Figure 6. Up to 950 ^{3}H decays per cell were tolerated as non-toxic. This result indicates that in excess of 10^{6} ^{3}H-adducts per cell would be required to produce any cell inactivation over this time.

Similar periods of accumulation of decays were studied for ^{125}I-AZR incorporated EMT-6 cells. ^{125}I-AZR was bound to cells at a level which would produce 40 decays/hour/cell on average. The cell killing observed by incorporated ^{125}I-AZR was indistinguishable from the killing

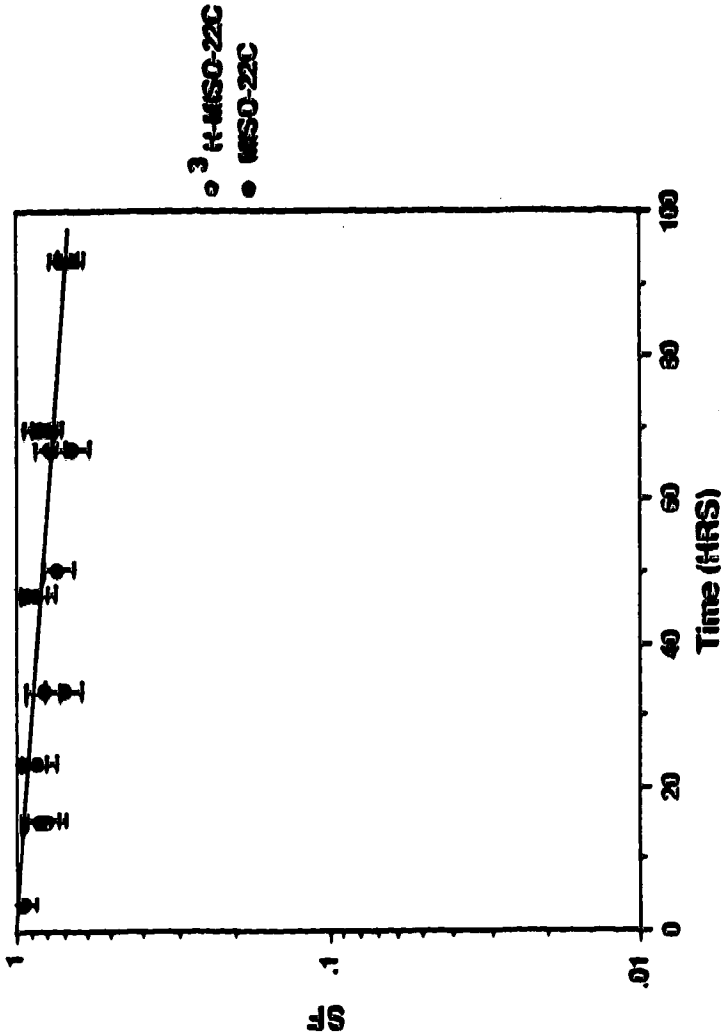

Figure 5: Following an accumulation of radioisotope decays for up to 4 days at 22°C, EMT-6 cells incorporated with ^{3}H-MISO and cold MISO were plated for survival assays respectively.

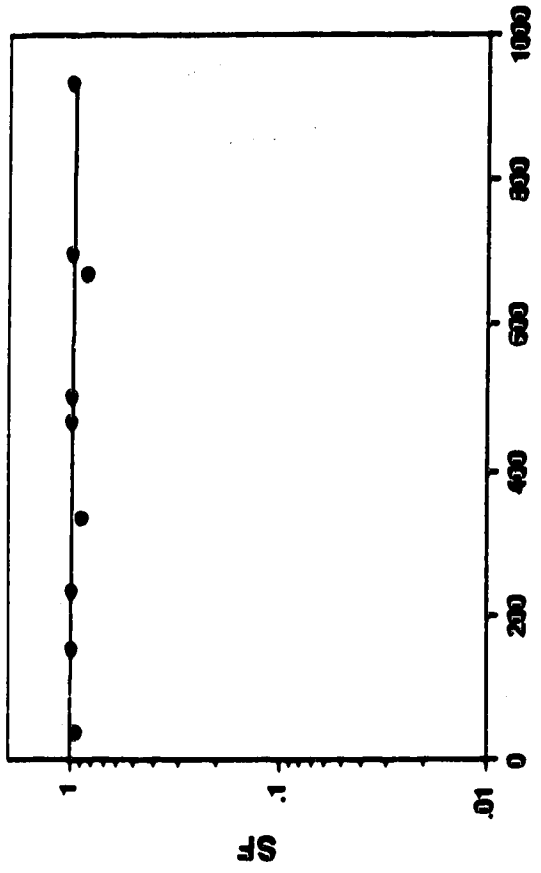

Figure 6: EMT-6 Cell Killing by Incorporated Tritium at 22C

observed by the toxicity of cold I-AZR alone (Figure 7). The difference of the survival fractions between the hot and cold I-AZR was plotted against decays per cell in Figure 8. Up to 1700 ^{125}I decays were tolerated as non-toxic. Under these conditions of cell storage, in excess of 10^5 ^{125}I-adducts/cell are required to produce cell killing.

Radiosensitivity is dependent upon cell proliferation status as well as upon inherent radiosensitivity and repair capacity. The method employed in this study involved the accumulation of potentially lethal lesions in single cells at 22°C when cell cycle progression is stopped or very slow. Lethal damage should have been expressed when cells were returned to 37°C. However, our results suggest that the repair capacity of EMT-6 cells at 22°C is adequate to circumvent lesions accumulated by these decays. Further suppression of the repair capacity was indicated as necessary to observe cell killing by these levels of isotope decay.

There have been many reviews dealing with various aspects of DNA repair after exposure to ionizing radiation. But essential to all the repair processes is a functioning enzyme system (51-53). It is well documented that enzymatic activity is temperature dependent because of the biochemical nature of the process. The equation Q10 = 2 predicts a decrease in enzymatic reaction rate by one half for every 10°C drop in temperature (61). Some studies suggest an even greater temperature dependence for repair of sublethal radiation damage. It is therefore reasonable to expect a minimum of 150% rise in DNA repair efficiency at the normal incubation temperature of 37°C when compared to that at 22°C. As our study showed that radioisotopes on sensitizer adducts are unlikely to produce sufficient cell kills to be useful in tumor treatments at 22°C, the killing efficiency would be even less at 37°C.

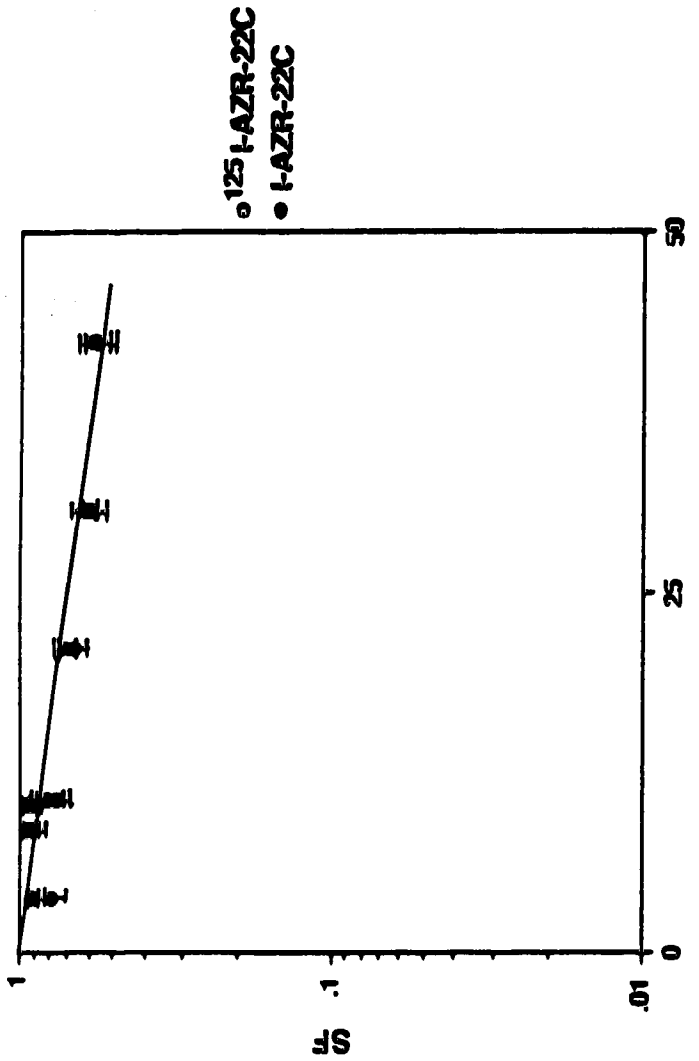

Figure 7: Following an accumulation of radioisotope decays for up to 2 days at 22°C, EMT-6 cells incorporated with ^{125}I-AZR and cold I-AZR were plated for survival assays respectively.

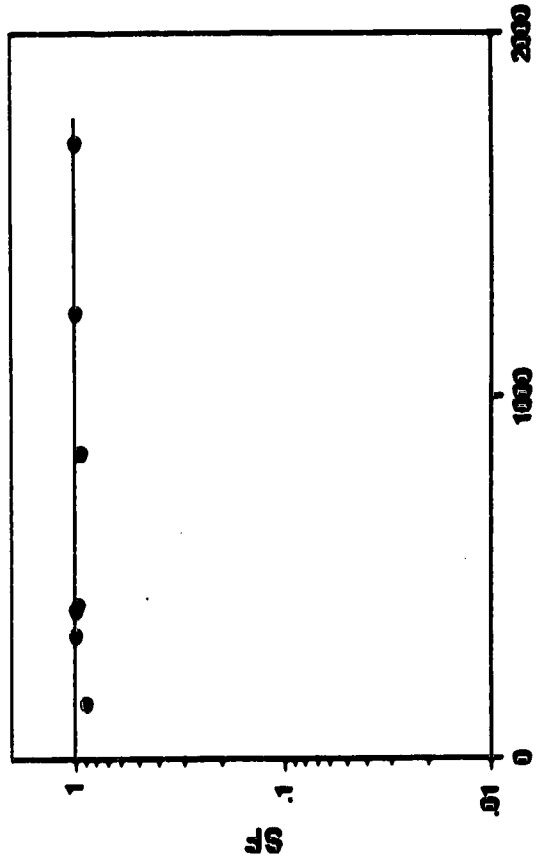

Figure 8: EMT-6 Cell Killing by Incorporated Iodine-125 at 22C

Most of the potentially lethal damage induced in mammalian cells by the levels of isotope decay encountered in this experiment will be overcome by cellular repair mechanisms at body temperature. It is also expected that the repair efficiency of DNA damage will decrease significantly or even be eliminated as temperature falls to 4°C. At subzero temperatures where no enzymatic repair of DNA is likely because of the frozen state, cell killing by the undisturbed accumulation of radioisotope decays might be even more effective.

The second study involved the storage of EMT-6 cells with incorporated radioisotopes ^{3}H or ^{125}I in suspension culture at 4°C and in 8% vol DMSO at -196°C. Various times were allowed to facilitate the accumulation of radioactive decays. Cells were then plated for colony-forming assays at 37°C. Unlike studies performed at 22°C, cell killing was observed for cells held at 4°C when little repair was possible. This was true for both ^{3}H (Figure 9) and ^{125}I (Figure 10). Cells frozen at -196°C in the presence of 8% vol DMSO to accumulate isotope decays, were found to be more resistant to radioactive inactivation than cells in liquid at 4°C (Figures 11 and 12). An estimate of radiation inactivation by radioisotope decays alone can be made by substracting the killing observed with unlabelled control drug from that observed with the radiolabelled drug. In obtaining the difference of survival fractions by subtraction, we have assumed that the change was due to radiation alone and that no synergism in cell killing existed between the effect of low temperatures and radiation. These difference in the survival fractions versus decays per cell accumulated are plotted in Figures 13 and 14. These data demonstrate the greater cell sensitivity to isotope decays at 4°C than -196°C for both ^{3}H and ^{125}I isotopes.

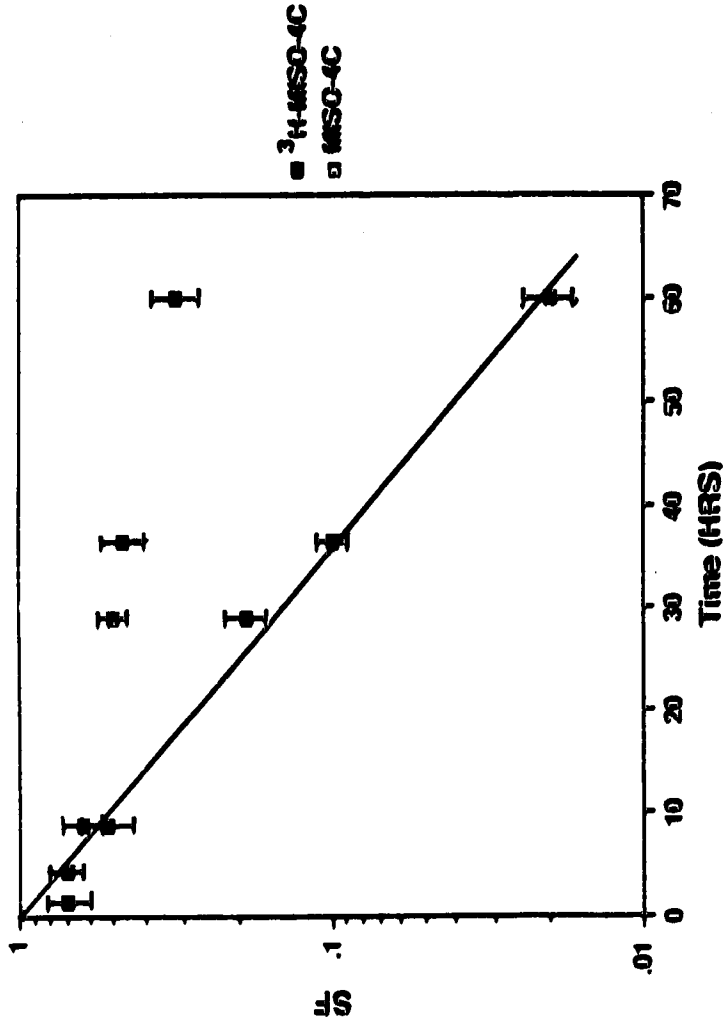

Figure 9: Following an accumulation of radioisotope decays for up to 60 hours at 4°C, EMT-6 cells incorporated with ^{3}H-MISO and cold MISO were plated for survival assays respectively.

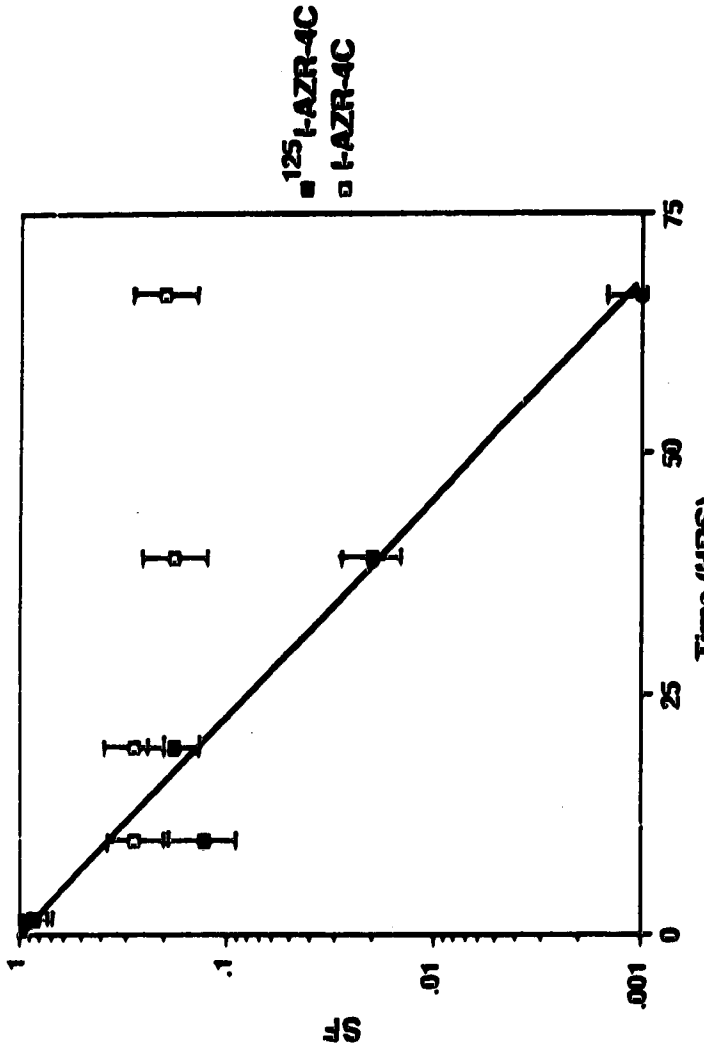

Figure 10: Following an accumulation of radioisotope decays for up to 65 hours at 4°C, EMT-6 cells incorporated with ^{125}I-AZR and cold I-AZR were plated for survival assays respectively.

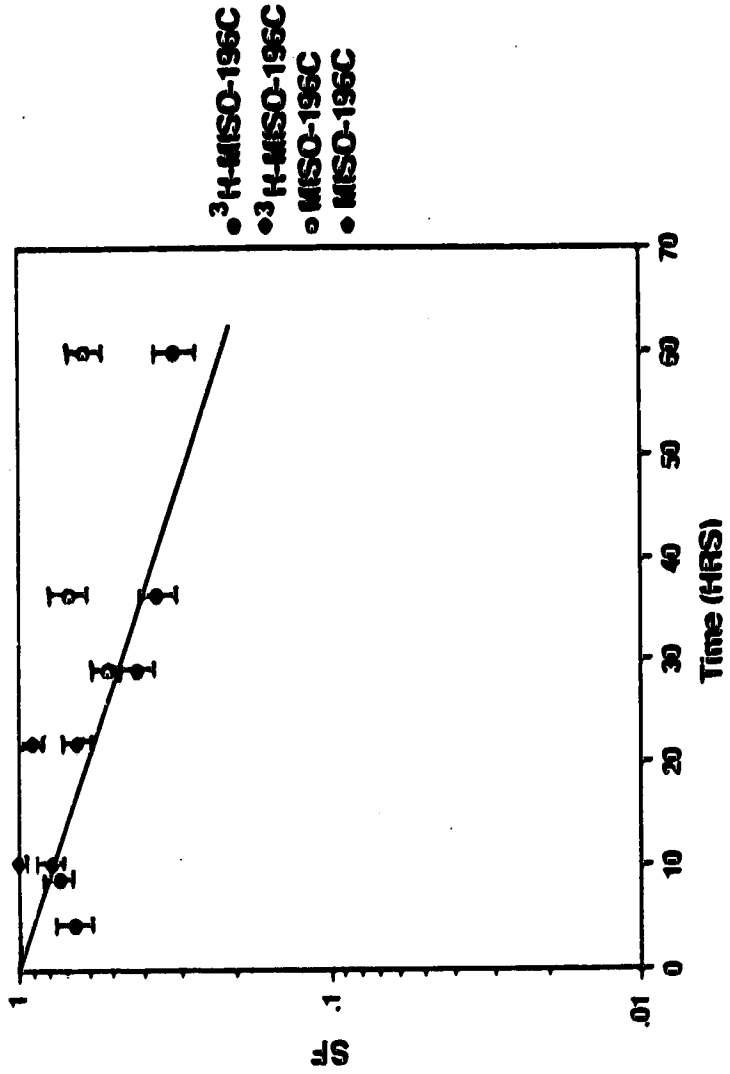

Figure 11: Following an accumulation of radioisotope decays for up to 60 hours at -196°C, EMT-6 cells incorporated with ^{3}H-MISO and cold MISO were plated for survival assays respectively.

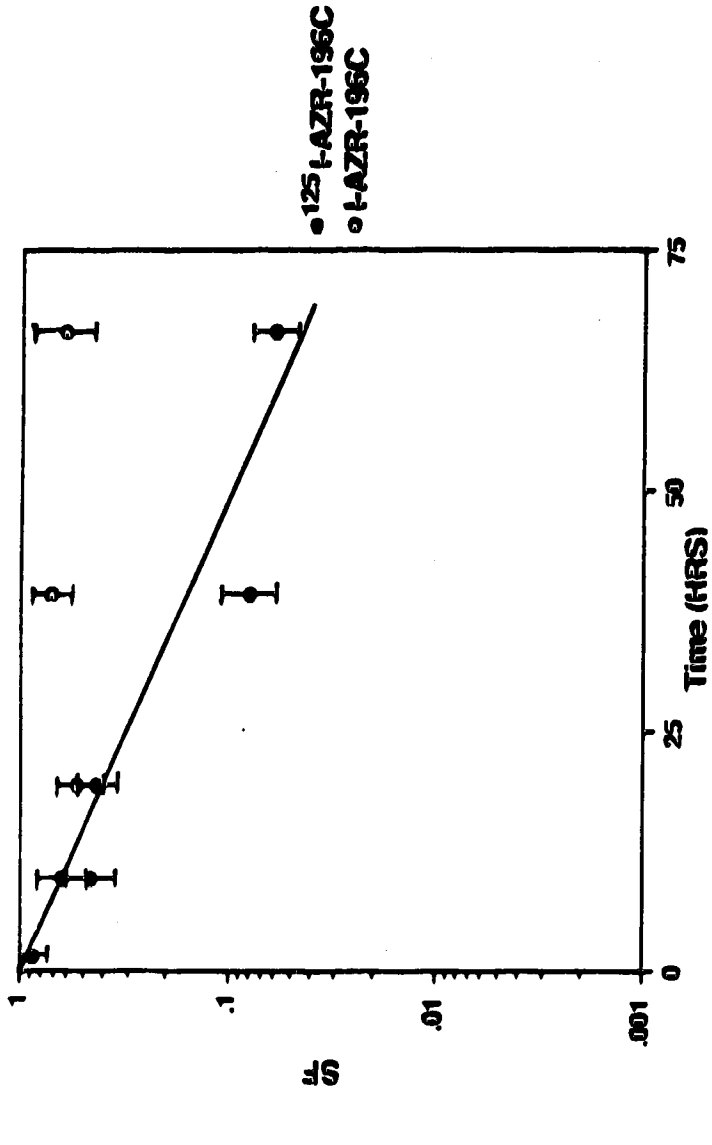

Figure 12: Following an accumulation of radioisotope decays for up to 65 hours at - 196°C, EMT-6 cells incorporated with ^{125}I-AZR and cold I-AZR were plated for survival assays respectively.

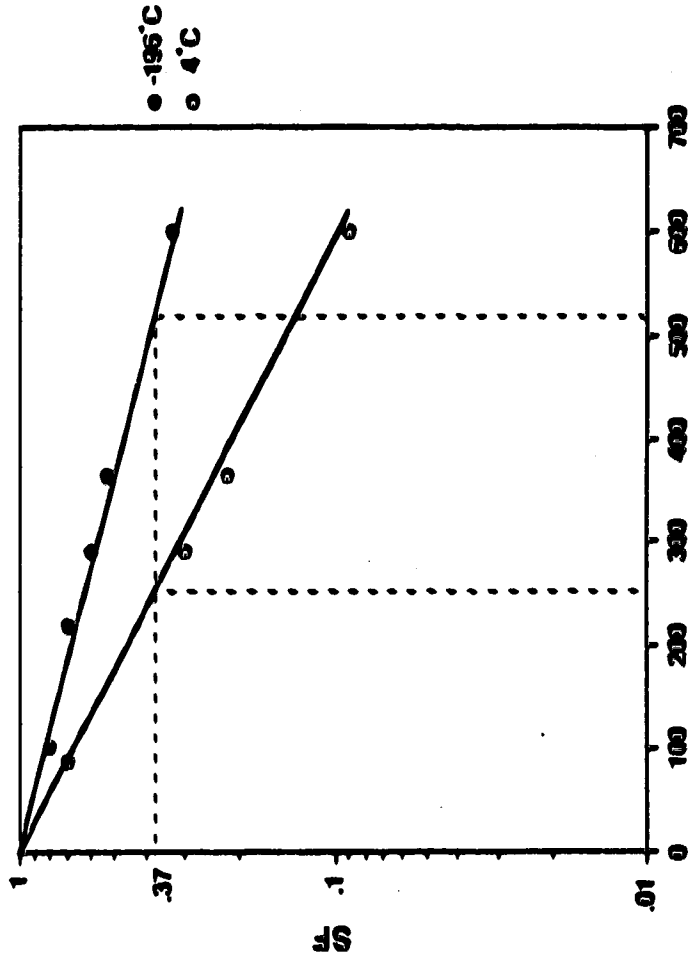

Figure 13: EMT-6 Cell Killing by Incorporated Tritium at 4°C & -196°C

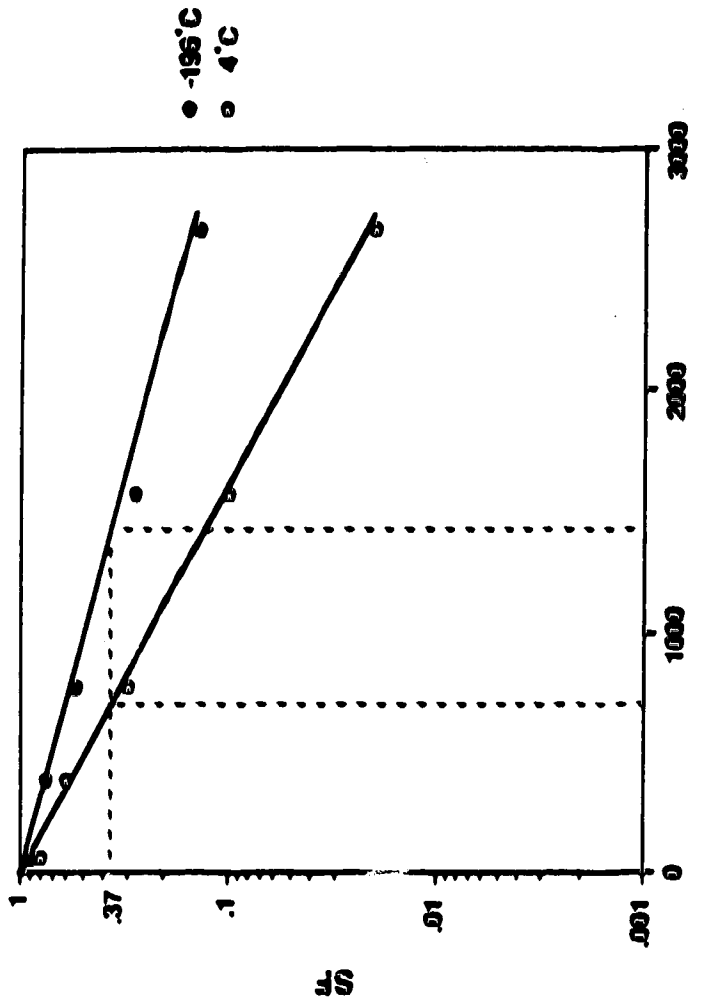

Figure 14: EMT-6 Cell Killing by Incorporated Iodine-125 at 4°C & -196°C

In order to express the biological effects quantitatively, D_o values have been computed. D_o is the dose required to reduce the surviving fraction to a value of 1/e (where e is the base of natural logarithms and 1/e equals 0.37). The values of D_o can be read off graphs as shown in Figures 13 and 14. Notice that the cell kill in these figures are due to the incorporated isotopes only. The D_o values are summarized in Table 3.

The number of ^3H decay per cell required to achieve a 63% cell kill at -196°C is 520. Taking into account the mean range of β^- particles (1 μm) emitted from the isotope, the actual percentage of total decays/cell that affect the nucleus or DNA structure will be 21% as calculated before. This is equivalent to an effective D_o value of 109 and is in reasonable agreement with data from Burki and Okada (32). They labelled mouse leukemia cells L5178Y with ^3H-thymidine and performed the suicide experiments at -196°C (41). Their D_o values for bifilarly labelled cells and unifilarly labelled cells are 80 and 167 respectively.

D_o values of decays/cell for ^{125}I are several folds higher than that of ^3H. This makes sense in light of the microdosimetry. If one considers the DNA molecule as the critical targets, only those ^{125}I situated within 0.02 μm distance are capable of delivering Auger electrons to the target for radiation damage. This range of 0.02 μm is the maximum. A recent microdosimetric study by Charton (54) of ^{125}I localized at the central axis of a DNA duplex and at distances from the axis, has demonstrated a sharp fall off in energy deposition approaching 0.004 μm from the decay point. This is in strong contrast to the mean range of 1 μm for the β^- particles emitted by tritium. These β^- particles are particularly powerful in causing ionization

TABLE 3

D_o (DECAYS/CELL) VALUES FROM THE SURVIVAL CURVES OF EMT-6 CELL INACTIVATED BY ISOTOPES BOUND THROUGH SENSITIZER ADDUCTS

	22°C	4°C	-196°C
^{3}H	>>950	250	520
^{125}I	>>1700	680	1420

and excitation at the track end. So although auger electrons have higher LETs than the β^- particle, their radiation effectiveness in EMT-6 cells is limited by their range of travel and their small number in the vicinity of DNA molecules. It has been well established that iodine-125 can exert severe biological damage at both cellular as well as molecular levels, when incorporated into the DNA via the thymidine analogue, 5-[^{125}I]-iododeoxyuridine (38). The D_o value for DNA-incorporated ^{125}I is in the range of 60 decays (64).

The observation that different killing efficiencies for isotope decay in cells at -196°C and at 4°C is not surprising. When any form of radiation (x-rays, gamma rays or charged particles) is absorbed in biological material, there is a possibility that it will interact directly with the critical targets in the cells. Alternatively, the radiation may interact with other atoms or molecules in the cell (particularly water) to produce free radicals which are able to diffuse and damage the critical targets. This is called the indirect action of radiation. As this indirect effect necessarily involves the intracellular environment, it is understandable that a change in the physical state of the cell at -196°C could affect the extent of the indirect action of radiation.

Preservation of cells and tissues at low temperature is greatly enhanced by the presence of effective cryoprotectants. DMSO was the cryoprotectant used in this study because of its low toxicity and relatively high permeability to cells. However, like most other cryoprotectants, e.g. glycerols and glycols, its also has the property of protecting cells from damage caused by ionizing radiation (55). Since our studies were performed with cells in the presence of both

frozen state and DMSO, we performed additional studies with external beam irradiation to determine the relative importance of DMSO and frozen state in reducing cellular radiation damage.

CHAPTER 5

EXTERNAL BEAM IRRADIATION OF CELLS IN LIQUID VERSUS FROZEN STATE

In the years immediately following World War II, in the wake of the first use of atomic weapons, research was conducted to identify a drug that would protect against ionizing radiation. Gray et al. (1) first documented oxygen as a factor of importance in clinical settings. The ratio of doses under hypoxic and aerated conditions needed to achieve the same level of biological effect is defined as the oxygen enhancement ratio (O.E.R). At high doses, the O.E.R. has a value of between 2.5 and 3 for most mammalian cells. For oxygen to act as a sensitizer, it must be present at the time of the radiation exposure, or at least during the lifetime of the free radicals that are involved, which is about 10^{-5} seconds. The oxygen fixation hypothesis was put forward to account for the sensitizing action of oxygen. It was postulated (56) that the reactions are as follows:

$$\text{DNA} + \text{irradiation} \xrightarrow{\text{deprotonation}} \text{DNA radical} + H^+ + e$$

$$\text{DNA radical} + O_2 \longrightarrow \text{lethal product (irreparable)}$$

$$\text{DNA radical} + \text{SH or } NH_2 \longrightarrow \text{DNA} + \text{SH or } NH_2 \text{ radical (repair)}$$

Hence the lack of oxygen results in a more efficient chemical repair within cells and less biological damage. In this study, the radioprotective effect of hypoxia is compared with that of other radiation modifiers.

Many radical scavengers, e.g., DMSO, have been described in chemical systems (55). Their ability to be effective radioprotectors of cells

depends upon radical scavenging at concentrations which are not toxic. Sulfhydryls are capable of interacting directly with products of water radiolysis as well as with radical sites on relevant biomolecules such as DNA (57). It was postulated that donation of hydrogen from the protector molecule to the radiation-induced bioradical (DNA-radical) results in repair of the damaged site. As well some SH compounds can infer some radioprotection by physiological mechanisms, such as reducing blood flow to tissue (60). The radioprotective effect of dithiothreitol, as representative of sulfhydryl compound, at a final concentration of 20 mM was compared with that of the other radiation modifiers.

Chemical modifiers of radiation effect discussed so far include DMSO, hypoxia and dithiothreitol. Their suggested mechanisms of radioprotective action are dependent upon diffusion of these chemicals through cellular water. If such proposed mechanisms are correct, it is conceivable that their effectiveness in a frozen phase would be greatly diminished. In this study, we have measured the radioprotective effect of frozen phase with and without chemical modifiers, at a temperature of -196°C. DMSO at 8% volume final concentration was added in all cases for a uniform cryoprotection.

No difference in cell survival was observed when EMT-6 cells were irradiated in suspensions without DMSO by gamma rays of Co^{60} at 22°C and 4°C. The addition of 8% vol DMSO showed a radioprotective effect resulting in a dose reduction factor (D.R.F.) of 2.1 at the 0.1 survival level (Figure 15). Standard errors in the figures were calculated by the formula SF x $(\frac{\sqrt{N}}{N} \times 100\%)$ where SF is the survival fraction and N is the average number of formed colonies per plate. Irradiation of the cells in frozen phase at -30°C (approximate) and at -196°C with

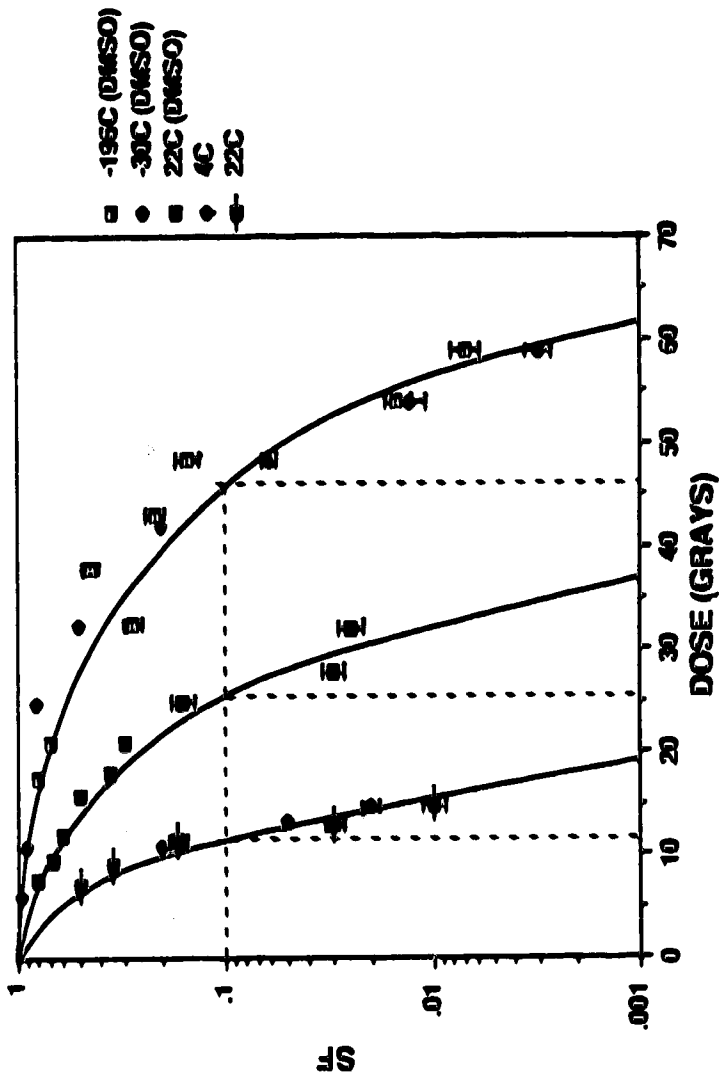

Figure 15: Low Temperature Radiation Survival Curves With & Without DMSO

8% vol DMSO resulted in a D.R.F. of 3.8 at the 0.1 survival level compared to unprotected cells at 22°C.

Irradiation of EMT-6 cells in the absence of oxygen or with 20 mM dithiothrietol in addition to the 8% vol DMSO at 4°C resulted in more radioprotection than that by DMSO alone (Figure 16). The D.R.F. for the sulfhydryl compound plus DMSO is greater than that of hypoxia plus DMSO, i.e., 3.0 vs 2.3. But EMT-6 cells irradiated at -196°C showed a similar resistant survival curve independent of the presence or absence of oxygen or DTT (Figure 17). These data suggest that there is a minimum rate of EMT-6 cell killing which is independent of water radical mobility and/or presence of radical modifying substance. Conversely, nearly 80% of the cell killing observed in oxygenated cells at 22°C involves diffusible free radicals.

A reasonable way to describe survival curves is to apply the linear-quadratic model and determine the α and β parameters. One can manipulate the linear-quadratic equation as follows, and plot $\frac{\ln S/So}{D}$ versus dose to obtain a linear response.

$$S/So = e^{-(\alpha D + \beta D^2)}$$

$$\ln S/So = -(\alpha D + \beta D^2)$$

$$-\frac{\ln S/So}{D} = \alpha + \beta D$$

When $-\frac{\ln S/So}{D}$ is plotted against D (Figure 18) the y-intercept value is α and the slope of the graph is β. These values are listed in Table 4. The five numbered graphs in Figure 18 corresponds to the numbered curves in Figure 17. Because values of α are so small, precise variations among linear plots cannot be determined. One can also see that in the

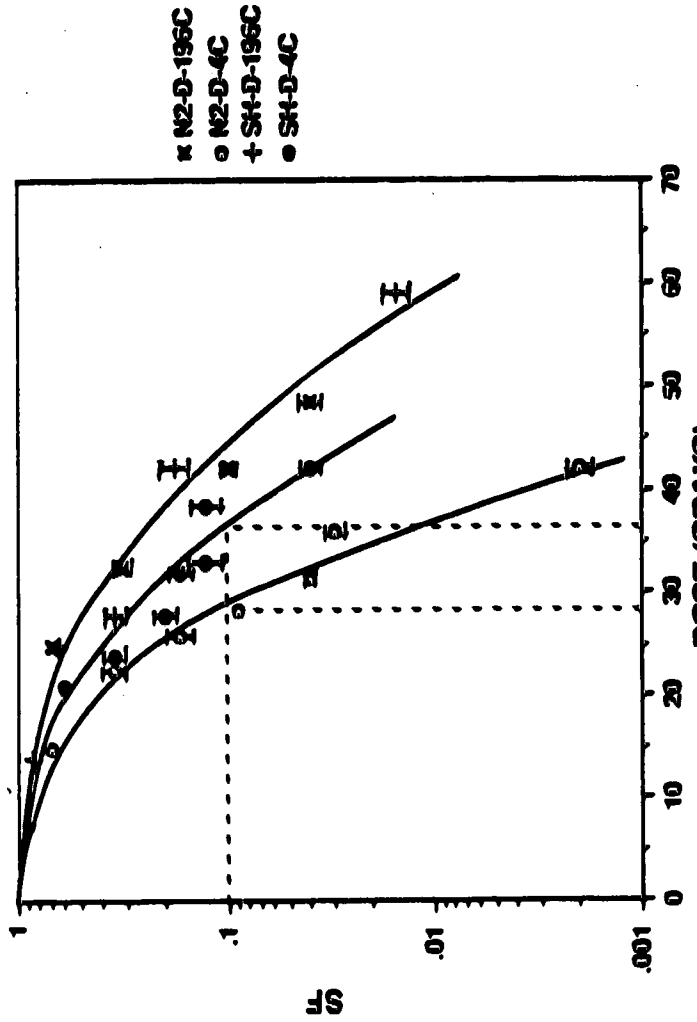

Figure 16: Low Temperature Radiation Survival Curves with Hypoxia or Dithiothrietol

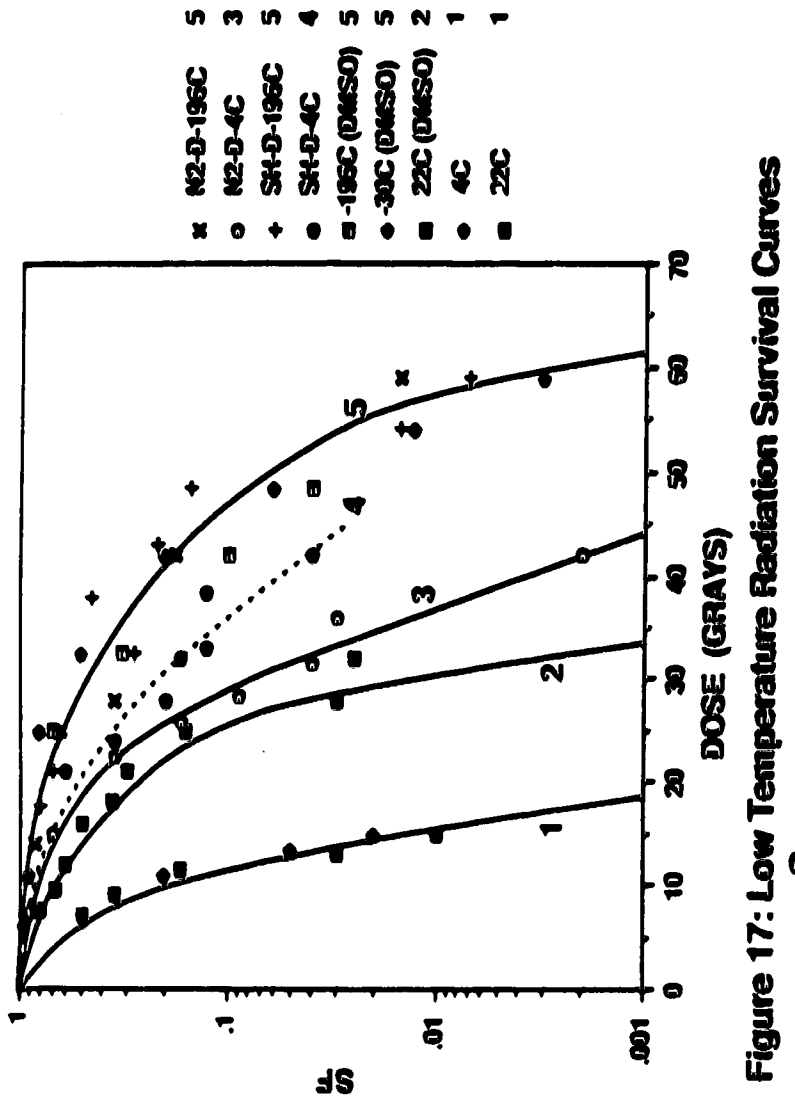

Figure 17: Low Temperature Radiation Survival Curves - Summary

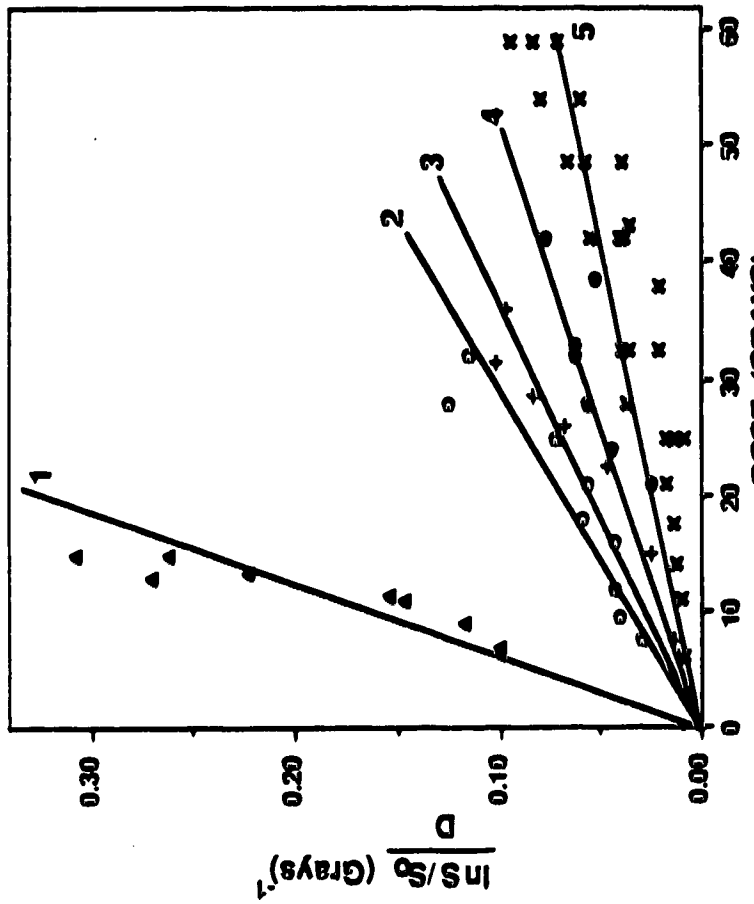

Figure 18: Low Temperature Radiation Linear Plot
(Computer Best Fits)

TABLE 4

α AND β VALUES OF SURVIVAL CURVES FOR EMT-6 CELLS IRRADIATED IN LIQUID AND FROZEN STATE

	α (Gy^{-1})	β (Gy^{-2})
1	0	0.0170
2	0	0.0035
3	0	0.0027
4	0	0.0017
5	0	0.0011

presence of radioprotectors and/or frozen state, cells become more resistant to radiation, and the effectiveness of the quadratic component of cell killing is reduced. Value of β is the smallest for the frozen state.

It has been said that the radioprotection effect of protectors on cells increases with increasing concentration until a plateau is reached. With DMSO, a maximum effectiveness is achieved with about 2 mol dl^3 (molar). It may be assumed that OH radicals are completely scavenged at this concentration. On this assumption, Chapman et al. (56) interpreted their results on protection of Chinese hamster fibroblasts by DMSO as a method of measuring the contribution of OH radicals to the overall lethal effect of radiation. The 8% vol. concentration of DMSO used in this study is equivalent to only 1.13 mol dm^{-3}, additional OH radical scavengers such as DTT might add to the OH· scavenging potential within these cells.

The observation that there is a similar cell survival from external radiation for cells held at -30°C (approximate) and -196°C is of importance. It has been shown by microscopic observations that an intracellular phase transition occurs at about -40 to -60°C in the presence of 8% DMSO (58). This implies that EMT-6 cells at -30°C will still contain some "liquid phase" water potentially capable of radical diffusion rates faster than in cells frozen to -196°C. Results from this study indicate that intracellular radical diffusion is slowed at -30°C enough so that further cooling may alter quality of intracellular "water/ice" but provides no additional protection.

Although dosimetry for incorporated radiosotopes was worked out in Chapter 2, it should be mentioned that the previous calculations

are suitable for cells suspended in liquid only. At subzero temperatures where extracellular ice formation occurs, water shifts down the osmotic gradient from intracellular compartments to extracellular compartments. This results is shrinkage of cells. The decrease in cell volumes could actually increase the density of the incorporated radioisotopes and subsequently the radition dosage to the DNA targets.

It should also be mentioned the the radioprotection observed for the ^{60}Co radiation might not be the same as that observed for the incorporated isotopes. This premise is based on the general findings that the α component of the α/β model tends to be dominant at the low dose range of 0 - 2 Grays and that the β component tends to be dominant at the higher dose range of 10 - 50 Grays.

CHAPTER 6

GENERAL DISCUSSIONS AND CONCLUSIONS

In an attempt to study the feasibility of tumor cell kill by intracellular radioisotopes, EMT-6 cells with incorporated ^{3}H-MISO or ^{125}I-AZR were assayed for cell survival after accumlating radioactive decays at different temperatures. Results demonstrate that at a temperature of 22°C and greater, EMT-6 cells can tolerate at least 950 ^{3}H and 1700 ^{125}I decays as non-toxic. The effective D_o value of 109 for the ^{3}H suicide experiments at -196°C correlates reasonably well with previous data (32) in the literature. The higher effectiveness of ^{3}H decays in comparison to ^{125}I decays in killing cells is not surprising. The high LET Auger electrons emitted from ^{125}I exerts a relatively large portion of its radiation effect through direct effect. Because of the short track lengths of the Auger electrons only those ^{125}I incorporated very close to the DNA molecule might be effective. This amount is minute when the small dimension of DNA (average dimension of 2 μm) and the non-specific distribution of ^{125}I-AZR throughout the cell volume are considered. β^- particles emitted from tritium have a relatively low LET and their radiation effects at low dose rate will result from both single- and double-particle events. It has been recently shown that the location of ^{125}I in relationship to DNA is critical in its radiation effectiveness. Therapeutic studies with high specific activity 6-[^{125}I]iodoMNDP has demonstrated that this Auger-emitting drug is effective in treating mice with a transplanted CMT-93 rectal adenocarcinoma, its efficacy being attributed to the selective intranuclear localization of 6-[^{125}I]-iodo-MNDP (59).

EMT-6 cells were found to be more sensitive to isotope decays at lower temperature. However, for the same number of accumulated decays, cells in frozen state are damaged less than the cells in suspension. The relative radiation effectiveness in liquid and frozen state is demonstrated again in the second part of the study using gamma rays. The change of state likely substantially decreases the diffusion rate of transient chemical species produced by radiation in the cellular water. These transient species are essential in eliciting the indirect effect of radiation on cells. This study further supports the thesis that at approximately -30°C, the diffusion of radicals are slowed enough so that continuous drop in temperature to -196°C gives no significant additional effects on the radical diffusion rates.

It is known that the transient radical species produced in cellular targets at 10^{-8} to 10^{-6} second can be modified by reactions with molecules of the cellular milieu over times for 10^{-6} to 10^{0} second (56). The radioprotective effect induced by the presence of radical scavengers in this study is in accordance with this mechanism. The radioprotective mechanism is illustrated by the α, β analysis where the two particleevents in cell killing expressed as β values decrease progressively as radiation protection increases. The near zero α values for the cell survival curves are approximations only. The values are too close for the determination of variations.

Maximum radioprotection is seen in the frozen state. The large extent of radioprotection imparted by hydroxyl radical scavengers suggests that OH· play a major role in aerobic cell killing at 22°C. It appears that they also play a significant role in cell killing by decays of incorporated isotopes. It also appears that a minor portion

of cell killing is independent of water radical or its mobility.

At 37°C when DNA repair is most efficient, even greater amounts of incorporated radioisotopes will be tolerated since the enzymatic processes associated with the detection and removal of damage and the resynthesis and ligation of DNA are temperature dependent. There is a growing opinion that this enzymatic repair capacity is variable in different cell types and results in different radiosensitivies.

If radioisotopes are distributed inside tumor cells as in the study, in excess of 10^6 ^3H and 10^5 ^{125}I-adducts/cell are required for radiation inactivation. It is most likely that the proposed suicide technique using MISO and AZR labelled with tritium and/or iodine-125 will not be useful in tumor therapy. If isotopes are to be successfully delivered to tumor cells on antibodies for therapy, it might be expected that at least 10^5 - 10^6 of ^3H or ^{125}I will be necessary. This is near the maximum quantity of monoclonal antibody/cell that may adhere to some tumor cells (36). Unless the chosen MoAb has a synergistic effect with the radioisotopes or a different intracellular distribution, e.g., internalization of MoAb to DNA structure, this study suggests that the radioeffectiveness of radiolabelled tumor-specific antibodies using ^{125}I- and ^3H will be minimal. Radiolabelled hypoxic cell sensitizers employed in this study might also be relevant for diagnostic use in clinical situations. On linking a gamma emitting radioisotope to hypoxic cells, nuclear scans could be used to detect hypoxic tumor fractions. This non-invasive means of detecting tumor hypoxia could have a significant bearing on future radiation therapy planning.

CONCLUSIONS

1) These studies show that radioisotopes (tritium or iodine-125) on sensitizer adducts are unlikely to produce sufficient cell kill to be useful as adjunctive tumor treatment.

2) Cellular repair mechanisms at 37°C can overcome most of the potentially lethal damage induced in mammalian cells by the levels of isotope decay examined in this study.

3) The radiation effectiveness of radiolabelled tumor-specific antibodies could be similar to the labelled sensitizer adducts in the study.

4) Cells are more sensitive to isotope decay at lower temperatures, where repair processes are minimized.

5) For the same number of accumulated decays, cells in frozen state are damaged less than cells in suspension at 4°C, presumably by elimination of indirect effect.

6) The radioprotective effect in frozen state observed with high dose rate ^{60}Co γ-rays results mainly from the reduction of two-particle events, most likely through the immobilization of diffusable radicals.

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