Many experiments are being conducted to find compounds that offer radioprotection against radiation damage and that are also non-toxic. It is hopeful that in the future, research for this technology will benefit patients undergoing cancer treatment by reducing radiation damage to normal cells and therefore reducing short and long term side effects experienced from treatments.

Hamster cells were irradiated at doses of 60 and 120 rad, with and without Sphingosine-1-Phosphate mixed in with their growth medium. Post irradiation, it was observed that the S1P molecule seemed to have a radioprotective effect by decreasing the amount of cell death compared to the amount of cell death that occurred with the absence of the molecule. The results of this experiment will sent to Dr. Jon Tilly at Massachusetts General Hospital. Dr. Tilly is currently researching S1P as a possible radioprotector.
An Experiment on the Radioprotective Effects of Sphingosine-1-Phosphate on V-79 Hamster Lung Cells

by
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AN EXPERIMENT ON THE RADIOPROTECTIVE EFFECTS OF SPHINGOSINE-1-PHOSPHATE ON V-79 HAMSTER LUNG CELLS

INTRODUCTION

In this thesis, the radioprotective properties of Sphingosine-1-Phosphate (S1P) were evaluated. S1P is currently being researched by Massachusetts General Hospital. This work focuses on the use of Chinese hamster lung cells (V-79). They were irradiated with two doses of gamma rays with and without the S1P molecule present in their growth environment. The cells were allowed to proliferate almost two full generations post irradiation before being counted to see how many cells survived the radiation exposure. The goal of the research was to determine if there was less cell death from radiation damage with the molecule present.

The history of this research started in 1948, when the first compound with radioprotection properties, called cysteine, was discovered [28]. This interested the U.S. Army knowing that nuclear warfare was a potential threat. In 1959, the Army set up a developmental program that tested over 4,000 compounds. They looked for compounds that not only offered radioprotection, but were also non-toxic [28].
Between 1959 and the present day, researchers have continued testing many different compounds, hoping to find one that will reduce or block damage to living cells from radiation exposure.

In 1997, Dr. Jon Tilly, who is the director of the Vincent Center Reproductive Biology department and a 1999 Department of Defense research award recipient, and his research team out of Massachusetts General Hospital published their results on the S1P molecule as a possible radioprotector [2]. It had tested successfully in their experiments with mouse ovarian cells. They are currently creating a database of information that includes a wide range of cell lines that the S1P successfully protects from radiation damage. They are also trying to discover the exact mechanism of this action by the molecule. Dr. Tilly’s research provides future hopes of using this molecule on people who are receiving radiation treatments to lessen the radiation damage to normal cells [2]. His work has been published in Nature Medicine and Advance, along with being mentioned in news stories by CNN and MSNBC [29, 30].

The objectives of this experiment were to evaluate the effect of S1P on irradiated V-79 hamster lung cells. The results will be forwarded to Dr. Tilly at Massachusetts General Hospital.
BACKGROUND

V-79 CELLS

The V-79 Chinese Hamster lung cells were chosen for a few reasons. This type of cell line is easy to grow, maintain, it proliferates quickly, is widely used, it can be interpolated to human cells response, and has a short cell cycle time of 9 to 12 hours. This cell line is used in a large variety of tests. Researchers like using this cell line because its properties are well known and it is readily available. This particular cell line was established in the 1950s in Japan\(^1\). The V-79 cells have been used in a wide variety of research including cell metabolism, links between cancer and PCBs, avascular tumor growth, and genetics to name a few [31, 32, 33].

The process for culturing V-79 Chinese hamster cells begins with seeding the cells as a single suspension or layer into a culture flask. After determining the cell concentration in the flask, a known number of cells can be cultured into glass or plastic petri dishes for irradiation. An incubator, a refrigerator-looking compartment that works like an oven, is set to the temperature and atmosphere that simulates the cells natural environment. The incubator was set to 37 degrees Celsius. A tank of carbon dioxide with a hose connecting into the incubator had a regulation valve that allowed for the incubator’s atmosphere to contain oxygen with

\(^1\) Mahadevan, Brinda. <brinda.mahadevan@orst.edu> “Hamster Cells.” March 14-April 23, 2002. Personal e-mail.
five percent carbon dioxide. The temperature and carbon dioxide levels were digitally displayed on the incubator’s control panel.

The V-79 cells grow in culture as adherent monolayers, meaning they grow as a single suspension and adhere to the surface of the container they are being cultured in. The spreading of cells into a monolayer is believed to be due to a secretion of extracellular matrix proteins and proteoglycans by the cell [3].

The V-79 cells are mammalian cells, which means they propagate and proliferate by mitosis. When a cell divides, it forms two identical daughter cells if outside influences (such as radiation) do not disturb the chromosome structure in a way that it does not correctly repair. The time required for a cell to divide and create two daughter cells is known as the mitotic-cycle time or the cell-cycle time [4]. As mentioned above, the V-79 cells have a cell-cycle time of 9 to 12 hours. When watching the cells by eye or with a 10x light microscope, the increase in cell colony size can be observed, which indicates the cells are proliferating.

The four phases of the cell cycle in order of occurrence are mitosis or the division phase (M) lasting approximately one hour, the pre-DNA synthesis phase (G1) lasting one hour, the DNA synthesis phase (S phase) lasting around six hours, and the post-DNA synthesis phase (G2) lasting approximately three hours (See Figure 1) [5]. The variation in the cell-cycle time for the V-79 cells is due to an occasional
delay in the G1 phase. The G1, S, and G2 phases of the cell cycle are the components of interphase, the period of cell growth that occurs before mitosis [27].

Since V-79 cells are a monolayer culture, they round up and become slightly detached from the culture flask just before mitosis occurs. The whole process of mitosis is the rounding up of the cells, the chromosomes condense, and finally the cells divide and reattach to the flask surface.

Figure 1. Phases of the cell-cycle for V-79 cells

The V-79 cells experience three major phases following their initial seeding (placement into the flask growth environment). The lag phase is a five-day period where a small amount of growth is observed after the cells are seeded into the
flasks. The lag is due to the cells being frozen in their cycle, getting used to their new environment. The Log Phase is a period of time that follows the lag phase in which an increase in cell number is observed. The length of the log phase depends on factors like seeding density, cell growth rate, and cell proliferation rate. The Plateau Phase follows the log phase. In this phase the growth rate of the culture decreases or proliferation stops completely because there is no longer any available surface to grow and the cells are touching each other. This phase was not important in this work, but out of personal interest, the cells were allowed to reach this phase in a few instances after the experiment to observe the characteristic effects.

The V-79 cell’s most widely used in vitro growth environment is McCoy’s 5A medium, which contains a formulation of inorganic salts, vitamins, amino acids, and other nutrients needed. A complete list of components is found in appendix D.

The amino acid concentration of the medium limits the maximum cell concentration along with cell survival and growth. One of the amino acids in the medium, glutamine, is very important because it is an energy and carbon source for the cells. Vitamins mainly affect cell growth and survival. The salts control the osmolality of the medium, regulate membrane potential, and regulate intracellular charge. Glucose is inserted into most media as an energy source [6]. The medium

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contains a 10% concentration of Fetal Bovine Serum (FBS). FBS consists of polypeptides, hormones, nutrients, metabolites, minerals, inhibitors, and proteins that transport minerals, fatty acids, and hormones. Most importantly, FBS inhibits the action of trypsin, a proteolytic enzyme that breaks the bonds between the cells and the medium. Trypsin's action creates the single suspension of cells.

Cultured cells are subjected to a series of tests to ensure they are performing as expected. Conducting a plating efficiency test is the best way to make sure that the medium and the growth atmosphere are suitable for the cell line. This test will detect minor deficiencies in the medium and minor amounts of toxins that might be present from contamination. Historically, a healthy V-79 cell culture has a reproducible plating efficiency of approximately 70%. This means that out of 100 cells seeded, 70 colonies will form and approximately 30 will have failed to live and form colonies.

A second test done to assess cell health is a growth curve test. For this research, this test was done by the biophysics laboratory staff before the cells were ready for experimentation. After they seeded the cells into two flasks, they watched for a lag phase of about 4 days before the cells started to produce colonies.

---

A third test involves calculating the surviving fraction of cells. The surviving fraction is the ratio of colonies produced to cells plated (100 cells per dish were plated in this experiment) with a correction factor for the plating efficiency (70% for V-79 cells), since not all cells that are plated grow into colonies. Surviving fraction is represented by the equation:

\[
\text{Surviving Fraction} = \frac{\text{Colonies counted}}{\text{Cells seeded} \times \left(\frac{\text{Plating efficiency}}{100}\right)}
\]

Viscosity of the cell solution is another parameter of importance. The viscosity of the culture is maintained mostly by the amount of serum present in the medium and it has little effect on cell growth. The viscosity is important when it comes to disaggregation, the process of detaching the cell monolayer from the flask surface with trypsin. If there is too much cell damage from the disaggregation process, then a less viscous medium must be prepared.

The V-79 cells grow well at a pH of 7.4. Any changes in pH can be viewed by watching the color of the medium. The medium is a pinkish/red color at an optimal pH and will become orange and then yellow as the pH falls. The color turns dark pink and then purple when the pH is increasing. A small sample of normal medium was placed in a 10mL beaker as a reference to compare the healthy color to the color of the medium in the cell cultures.
The significant gases used for maintaining the cell culture in the incubator were oxygen and carbon dioxide. Carbon dioxide is said to have a complex role in maintaining cultures. The amount of carbon dioxide in the atmosphere will regulate the amount of carbon dioxide that is dissolved in the medium as a function of temperature. If more than 2 mm of medium per petri dish would have been used, diffusion of the oxygen and carbon dioxide through the culture would be decreased.

The atmospheric temperature has a direct effect on cell growth by influencing the pH of the culture because there is an increase in the solubility of carbon dioxide as the temperature decreases. The heat dial on the incubator was turned up five degrees before opening the door because a few degrees of heat is lost while the door is open and the lost heat is slow to recover. The CO₂ level was increased before opening the incubator door by turning up the inlet valve of the attached CO₂ canister. This step avoids a drop in the incubator’s atmospheric carbon dioxide while the door is open.

It is important to change the medium of the flaked cells every 3 days followed by a subculture, a process that decreases cell density, so that the cells won’t overgrow and exhaust the medium too quickly. There are four factors that signal a need for a change in the medium. The first factor is a drop in the pH of the medium. This can be viewed by eye because the medium will start turning an orange color.
The second factor is whether or not the cells proliferate at a high rate. The V-79 cells have a fast reproduction rate of 9 to 12 hours, which is why the medium is changed every three days. Slower growing cultures only require a medium change once every week. If the cells are exhausting the medium, a change in color from the drop in pH will occur.

The third factor is the cell type. Some cell types stop dividing at high cell densities. The V-79 cells stop dividing when they start dying from lack of nutrients. Finally, the fourth factor is cell morphology. When checking the petri dish cultures twice a day, a granular appearance around the nucleus or clumps of cells floating in the medium must be watched for because these are warnings that the cells are dying.

Subculturing of the cells was done twice. The protocol was to remove the medium, and dissociate the monolayer with trypsin. The way in which the cells attach in their monolayer and adhere to the flask is mediated by cell surface glycoproteins, Ca\(^{2+}\) ions, and various other proteins and proteoglycans [7]. The trypsin works by degrading these attachments. Enough trypsin was used to cover the culture with a thin 2-mm layer. The trypsin only needs to set with the cells for about 30 seconds before the monolayer begins to dissociate from the flask. Fresh medium with serum is then added by repeated pipetting to make sure it is evenly distributed throughout the culture. This is where the 10% FBS is important since it inhibits the effect of
trypsin on the cells. A split ratio of 1:10 is used for V-79 cells meaning for every one cell that was in the flask three days prior, ten must be removed. After adding fresh growth medium to the flask, a pipette is used to withdraw (0.5ml) the cells. The cells are put into a new flask with 2-mm of fresh medium, and continue proliferating.

**GENERAL CELL MECHANICS**

The cell cycle is regulated by cyclin-dependent kinases (CDKs). Each CDK has a particular cyclin (a protein) working with it. The CDKs and cyclins produce protein substrates that run the events of the cell cycle. The CDKs are numbered one through six and the cyclins are alphabetized A through E. CDKs do not operate on their own, they need to be paired with a cyclin in order to be active along with phosphorylation of a threonine residue. CDKs can have their activity stopped by phosphorylation of a tyrosine residue, which is located on the adenosine triphosphate-binding area. An inhibitory protein can also stop their activity. When the cell cycle has completed, the CDKs are inactivated by ubiquitin-mediated deterioration of the cyclin. When a cell goes into the S phase for example, this is regulated by cyclins D,E, and A pairing with CDK4 and CDK2. The D cyclins are growth factor sensors [8].

Molecular checkpoint genes also mediate the cell cycle. Checkpoint genes have the role of making sure the cell cycle’s events occur in correct order. The G2 phase of
the cell cycle is considered one of the more radiosensitive of all of the phases [9]. A checkpoint gene stops the cycle in this phase to delete chromosomes that contain damage before it allows the cycle to continue on to the mitosis or division phase. It is believed that there is also a checkpoint gene in the mitosis phase that checks the spindle function. If something is not correct in this part of the phase, mitosis is stopped. If a chromosome with radiation damage passes through the checkpoint, errors in the chromosome’s role during mitosis could signal the formation of cancer or mutation in the future [9].

LABORATORY ASEPSIS

It is important to keep asepsis in the laboratory. Even slight impurities in the cell cultures could kill them or create false results. Most toxins are difficult to detect until they are already deteriorating the culture. All pipettes, flasks, and petri dishes were washed prior to use. The incubator was located in a low traffic area, which decreases the chance of introducing dust, spores, and drafts that carry them into the cultures. It is also best to keep the media, stains, and other required chemicals that go into the culture free from contamination, and to keep them stored at their specified temperatures. When transporting the cells between the lab and the irradiator, they were placed into a portable Styrofoam incubator.
**OXYGEN ENHANCEMENT RATIO**

The oxygen enhancement ratio (OER) is the ratio of radiation doses required to achieve the same biological damage in hypoxic and aerated cells. If oxygen was present in the cells during irradiation, free radicals that combined with the oxygen could have formed an organic peroxide which results in making any damage to DNA permanent. If oxygen were not present, the DNA could repair itself and function normally. As the oxygen concentration in a cell increases, the cell becomes more radiosensitive [11]. The OER has been shown to vary according to a cell's position in the cell cycle [10]. The V-79 cells used in this study were an asynchronous culture, meaning the cells were distributed along all phases of their life cycle. This means that the cells were at different levels of radiosensitivity. Cells that are in the S phase tend to have a higher OER than the cells in the G1 or G2 phases [10].

**GAMMA RADIATION**

Gamma radiation is classified as a sparsely ionizing radiation. The energy of gamma radiation reduces its intensity as it passes through increasingly thick absorbers, but it is never completely absorbed.
RADIATION DAMAGE

Gamma radiation is an indirectly ionizing electromagnetic radiation. This means that it does not produce chemical change or biologic damage by itself, but it gives up its energy when it is absorbed into a medium, causing the production of fast moving, negatively charged particles.

The Compton interaction was the dominant way in which the gamma photons interacted with the cells because of the energies of the gamma rays [14]. With Compton interaction, a gamma photon interacts with a loosely bound electron. Part of the incident photon’s energy is given to the electron as kinetic energy; while the incident photon continues moving in a deflected path with what energy it has left. The incident photon’s energy has now been transferred to a fast electron and a photon with reduced energy. The fast electrons can ionize other atoms of the absorber and break chemical bonds, while the reduced energy photon continues interacting with atoms until it has expended all of its energy. Pair production was another likely way that the photons could have interacted with the cells. For this interaction to occur, the incident gamma ray must have a minimum energy of 1.022 MeV. This gamma ray interacts near the nucleus producing a negatron and a positron. The beta particle passes through the matter creating ion pairs until it becomes a free electron or apart of an atom. The positron collides with an electron, annihilating itself and the electron and emitting two 0.511 MeV gamma rays [15].
The photoelectric effect was not considered since that interaction occurs most in with low energy photons.

The biological damage from radiation mainly results from damage to DNA. When the gamma rays are absorbed in the medium, it is possible for them to directly interact with the DNA of the hamster lung cells. The radiation can also interact with other atoms, molecules, or water of the cell. When the radiation interacts with water, free radicals are formed and can damage DNA in a process called indirect action. A free radical is a free atom or molecule with an unpaired orbital electron in the outer shell, causing the atom to be unstable. Since 80% of the cell is water, there is a good chance that the photons will interact with a water molecule to become ionized. This process can be represented by:

\[ \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^+ + e^- \]

The ion radical \( \text{H}_2\text{O}^+ \) has a short lifespan of \( 10^{-10} \) seconds, and decays to form free radicals. The ion radical can react with another water molecule to form a hydroxyl radical, OH•, represented by:

\[ \text{H}_2\text{O}^+ + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+ + \text{OH}• \]
The hydroxyl radical has an unpaired electron, is unstable, and can damage the DNA of the cells. It is estimated that two-thirds of the damage from gamma irradiation to mammalian cell DNA comes from hydroxyl radicals [16].

The dose rate is one of the main factors that will determine what type of damage a cell will experience. Generally, less damage occurs when the dose rate is reduced and extended over a longer exposure time. The reduction in cell death is due to the repair of sublethal damage [17].

Three classifications of radiation damage exist: sublethal damage, potentially lethal damage, and lethal damage. Sublethal damage is normally repaired within hours after damage occurs. It can be seen mostly when cells are irradiated with a fractionated regimen rather than a single dose because time is given between doses for repair to occur. More cells tend to survive the fractionated regimen than when the same total dose is given in a regimen. Potentially lethal damage is damage that can be fixed by placing the cells in a special environment that promotes the cells to correct their damage and continue growing. Lastly, lethal damage is damage that cannot be reversed and leads to cell death [18].

When the cells were irradiated, damage to the cells were from single strand and double strand breaks in the DNA from direct or indirect damage. Direct damage is caused by the incident photon interacting directly with the DNA and indirect
damage is caused by the incident photon interacting with atoms in a cell, producing free radicals that may inflict DNA damage. Increasing the dose causes an increase in the number of strand breaks to the cells. The DNA in cells is somewhat resistant to radiation damage because of the natural presence of free radical absorbers and protection of the DNA by other structures in the cell. Single strand breaks are easily repaired. Double strand breaks can be repaired as well, but they are the most deadly break produced in chromosomes by radiation, due to the chromosomal aberration that it forms [19].

Chromosome aberrations result when a cell is irradiated early in interphase. The lethal aberrations that could occur are the dicentric, the ring, and the anaphase bridge. Each of these is described briefly below:

- **Dicentric:** A chromosome that has replicated to have two centromeres. This occurs when two separate, nearby chromosome breaks reattach to each other.

- **Ring:** A ring shaped chromosome is formed with one centromere. This forms when a break occurs in each arm of a chromatid. The nearby ends reattach to each other forming a ring.

- **Anaphase Bridge:** This occurs when there are breaks that occur late in the cell cycle (G2 phase) in both chromatids of the same chromosome and they reattach together. When anaphase occurs the chromatids stretch towards each pole, forming a bridge-like structure.
There are two other forms of chromosomal aberrations that are not lethal, but may lead to mutation or cancer in the cell. These aberrations are translocation and deletion (See Figure 2) [20]:

- Translocation has two broken ends of a chromosome reattach to the chromosome, except that they have swapped places.
- Deletion is a double break occurring in one leg of a chromosome. The end of the chromosome reattaches itself to the main body with the broken piece missing, and therefore it is deleted and free floating.

Figure 2. Non-lethal chromosome aberrations

Chinese hamster cells were among the best cells to use for this experiment because past experiments had already shown that they have a direct correlation between
aberrant chromosomes at the first post irradiation division followed by the failure of the cell to form colonies [21]. This means they die quickly if they are going to die at all.

**RADIOPROTECTION**

Radioprotectors are compounds that reduce the biologic effects of radiation. With the Chinese hamster lung cells, some possible ways in which SiP could have provided radioprotection are that the radicals reduced the oxygen concentration in the cells, which would lessen the cell’s sensitivity to radiation damage, and the absorption of the free radicals, which prevents the formation of oxygen-based free radicals or hydrogen-atom donation, which in turn aids in the repair of damaged DNA.

It was shown by experiment by Dr. Tilly’s team, that when SiP was given prior to cancer treatments (radiation or chemotherapy), less cell death of normal cells was observed post treatment. SiP blocks the activity of a molecule called ceremide that is associated with cell death. The ceramide-Bax-caspase pathway, which begins when a lipid molecule called sphingomyelin is converted into ceramide by an enzyme called sphingomyelinase (SMPD-1), causes the death of many types of cells in response to the cancer therapy [2].
The "sphingosin" backbone of sphingolipids was named in 1884 by J. L. W. Thudichum because of its Sphinx-looking appearance [1]. There has been much research on the role of sphingolipids in the signal transduction pathways that maintain cell growth, differentiation, multiple cell functions, and cell death. S1P along with other sphingolipid metabolites have been shown to modulate cell proliferation, and it has been suggested that is serves as a second messenger in cell proliferation and survival. Most of the S1P’s biological effects are due to binding to unidentified receptors on a cell’s surface (See Figure 3 by Avanti). In the following picture of the S1P molecule, the green represents carbon, the red represents oxygen, the pink represents phosphate, the white represents hydrogen, and the blue represents nitrogen. The chemical formula of S1P is C_{18}H_{38}NO_{5}P. The percent of composition of each element is carbon at 56.97%, hydrogen at 10.09%, nitrogen at 3.69%, oxygen at 21.08%, and phosphate at 8.16% [22].

Figure 3. S1P Molecule by Walter Shaw
MATERIALS AND METHODS

CELL CULTURING TECHNIQUES

The V-79 cells were obtained and cultured by the Oregon State University Biophysics/Biochemistry department. The cells were seeded as monolayers in two 75-cm² plastic flasks and cultured for five days in 2 mm of McCoy's 5a medium containing 10 percent fetal bovine serum (FBS). A hemocytometer was used to determine the cell concentration in the flasks. The cells were incubated at 37 degrees Celsius in a five percent carbon dioxide atmosphere. The pH of the medium remained at a stable 7.4 through the culture's life.

The flasks were horizontal when they were opened. It is best to keep them at the shallowest angle possible to keep the cells covered in growth medium. The petri dishes had loose fitting tops, allowing the warm atmosphere to ventilate through the culture. The caps on the flasks were also kept fairly loose for the same reason.

CELL IRRADIATION

One hour prior to irradiation, the cells were trypsinized and transferred to 35-mm plastic petri dishes with fresh medium. The 35-mm dish size was chosen because their size fit well in the irradiator chamber, and there was plenty of surface area for cell growth. Cells were put into twenty-one separate petri dishes, and each dish contained approximately 100 cells. One hundred cells per petri dish was a number
easy to count, and with the low cell density, they had room to spread out to create their colonies. Ten dishes contained the medium with the molecule, another eight dishes contained just the medium, and three dishes contained just the medium and were set aside for observation purposes. The S1P molecule was introduced into the medium by pipette, using one-eighth of a milliliter per dish and then swirling the dish for uniform distribution.

The cells were irradiated in the OSU cobalt-60 gamma irradiator (See Appendix A.2). The Linear Energy Transfer (LET) is a calculation of the amount of energy from charged particles transferred per unit length of track. The electrons liberated by Cobalt-60 gamma rays have an LET of 0.2 KeV per micrometer. Since Cobalt-60 has a low LET compared to other radiations, it is slightly less effective biologically than other forms of radiation (250 kV x-rays have an LET of 2 KeV per micrometer) [12]. Some other properties of Cobalt-60 include a 5.3-year half-life and 0.31 MeV beta at 100% abundance, 1.17 MeV at 99% abundance, and 1.33 MeV at 99% abundance gamma energies [11, 13]. The beta calculated to have a range of 0.082 g/cm² and was not considered in this experiment due to its absorption in the one millimeter thick polystyrene petri dishes (1.04 g/cm³ density) and the stainless steel irradiator casing (8.0 g/cm³ density) which is over 20.6cm in length and located between the source and the irradiation chamber. The minimum amount of stainless steel needed to absorb the beta was calculated to be 0.1mm, and
polystyrene it was 0.8mm. Therefore, the beta did not even reach the petri dish in the chamber.

Five dishes that contained the molecule were irradiated on the bottom shelf of the OSU cobalt-60 irradiator to a total dose of 60 rad (0.60 Gray), and the other five that contained the molecule were irradiated to 120 rad (1.2 Gray) (See Appendix A.1, A.2, A.3). Five dishes with no molecule were irradiated to 60 rad (0.60 Gray), and the last three with no molecule were irradiated to 120 rad (1.2 Gray). It took 3 seconds to irradiate to 60 rad (20 rad per second), and 5 seconds to irradiate to 120 rad (24 rad per second). The last three dishes of non-molecule medium were set aside for observation purposes to determine if the cells were in good health. The gamma doses of 60 and 120 rad were chosen because this is what has been used by past researchers who have irradiated Chinese hamster cells to study cell survival [23].

The biologic effect from the breaking of DNA chemical bonds resulted in cell death seen within 9 hours or less post radiation, depending on what phase of replication the cell was in. Since these cells have such a rapid turnover, the damage became evident very quickly.

A graph was used to show the link between radiation dose, the presence of a potential radioprotector, and the number of cells that survived as these factors were
altered (See Figures 3). It is seen that the cells suffer a reproductive death by failing to form colonies. The cells that did survive and continued to form colonies are termed clonogenic cells.

COUNTING SURVIVAL

The cells were returned to the incubator post-irradiation for 15 hours before counting. Fifteen hours was chosen for prime counting time because the life cycle of the hamster cells is 9 to 12 hours. Noticeable colonies of almost two generations are easier to count than one, if the cell survived irradiation. Mitotic death is the dominant form of death for cells attempting to divide after irradiation. The cells could have also died from programmed (apoptotic) cell death once the cell realized there was damage. In apoptosis, the cell dies usually for the good of the organism, without attempting to divide. In the literature it has been reported that a dose of 2 Gray (200 rad) or less is considered a lethal dose to proliferating tissues such as the Chinese hamster cells [24].

A short-term viability assay was performed on the cells to determine cell survival. This test is done after any trauma (like the irradiation experienced by the culture) to see how many cells survived. This test was preferred over a long-term viability test because it provides comparable results in a shorter period of time. The fraction of cells that were physiologically alive was determined by adding 50 microliters of 0.4 percent trypan blue solution to the dishes 15 hours post irradiation and then scoring
the 100 cells as stained or non-stained under a light microscope at 10x. The trypan blue viability test relies on the breakdown of the cell membrane post irradiation so that the dye can be taken up into the deteriorating, or already dead cells. If a cell is still in good living condition, the membrane does not allow the dye to permeate it. This test was a good choice since it not only displays the cells that are already dead, but it also shows the cells that are in the process of dying along with any colony that might have had time to form. The dye was added to the culture only at counting time and it was allowed to sit for 1 to 2 minutes before counting began. It is important to have the counting completed within 30 minutes of starting because the dye will start to deteriorate the viable cells around this time.

The petri dishes were placed under the microscope set on 10x at 1 to 2 minutes after adding the dye, and the number of stained cells was counted along with the total number of cells present. If a cell had created a colony from one life cycle, yet stained blue, it was counted as dead. If the cell had almost two generations of colonies or less, but still remained unstained, it was counted as living. Next, the surviving fraction was calculated to determine how many cells survived the irradiation. A felt tip pen was used to mark counted colonies along with a hand held counter.
RESULTS

Almost each test was conducted five times. A table of the counted results is shown below followed by a table containing the calculated surviving fractions.

Table 1. Number of surviving cells per 100 seeded

<table>
<thead>
<tr>
<th>Trial</th>
<th>Molecule 60 rad</th>
<th>No molecule 60 rad</th>
<th>Molecule 120 rad</th>
<th>No molecule 120 rad</th>
<th>Observation Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59</td>
<td>30</td>
<td>43</td>
<td>0</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>51</td>
<td>70</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>65</td>
<td>0</td>
<td>58</td>
<td>0</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>32</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>28</td>
<td>48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Surviving Fraction

<table>
<thead>
<tr>
<th>Trial</th>
<th>Molecule 60 rad</th>
<th>No molecule 60 rad</th>
<th>Molecule 120 rad</th>
<th>No molecule 120 rad</th>
<th>Observation cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.87</td>
<td>0.44</td>
<td>0.63</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>0.88</td>
<td>0.75</td>
<td>1.03</td>
<td>0.00</td>
<td>1.01</td>
</tr>
<tr>
<td>3</td>
<td>0.96</td>
<td>0.00</td>
<td>0.85</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>0.94</td>
<td>0.47</td>
<td>0.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.88</td>
<td>0.41</td>
<td>0.71</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This data presented below in graph form shows the radioprotective effect of the molecule:
Figure 4. Average Number of Surviving Cells by test type

![Graph showing average number of surviving cells](image)

As it can be seen, the plating efficiency of the observation cells remained around 70%, which is the plating efficiency established for this cell line.² It took three days to run all of the tests. Each day that new cultures were made, one new observation dish was made.

As the dose increased between the 60 rad and 120 rad cells, a difference in the amount of cells survival is observed. It is also observed between the 60 rad molecule cells (M60) and 60 rad non-molecule cells (O60) that the cells without the S1P molecule had a much lower survival (by 30%). The same effect is observed between the 120 rad molecule cells (M120) and 120 rad non-molecule cells (O120), where the non-molecule cells had an increase in death by almost 50%. It is

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observed between the observation cells and the irradiated cells, that the radiation treatment did induce radiation damage to the experimental cells.

The cells that had been treated with Si P and were irradiated at 60 rad (M60) had results that stayed approximately the same through all five trials. Approximately 59 to 65 cells survived in each test. The mean survival was calculated to be 61.6 cells per 100, with a calculated standard deviation of 2.7.

There was fluctuation in the results in the tests involving no Si P with the cells being irradiated at 60 rad (O60). Three of the five results were in the 70 percent range. One reason for the fluctuation could be attributed to what phase of the cell cycle the majority of the cells were in. The mean cell survival calculated to be 28.2 per 100, with a standard deviation of 18.3.

The two means of 61.6 and 28.2 representing cell survival for the M60 and O60 respectively, seem to clearly indicate that the O60 cell survival was statistically less. This observation was justified with a formal hypothesis test based on the sample statistics obtained from the surviving fraction results:

<table>
<thead>
<tr>
<th></th>
<th>M60</th>
<th>O60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Survival</td>
<td>0.906</td>
<td>0.414</td>
</tr>
<tr>
<td>Sample Size</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.0016</td>
<td>0.07</td>
</tr>
</tbody>
</table>
Since the two samples are independent, sample size does not exceed 30, they are normally distributed, and the variances are unknown and found to be unequal by the F-test, a t-test was conducted [25]. The t-test value of 16.4 calculated was within the critical region of 2.132 with 95% degree of confidence. It was concluded that there was sufficient evidence to support the claim that M60 mean cell survival is greater than the O60’s.

The cells that were irradiated at 120 rads with the molecule present (M120) had fluctuation in the results, but averaged to a 50 percent survival rate. The mean cell survival was calculated to be 55.8 cells per 100, with a standard deviation of 10.6. This is much improvement compared to the data shown for the cells that were also irradiated at 120 rads with no molecule (O120). Each test resulted in 100% fatality of the cells. Since the results of this test appeared predictable this test was only performed three times to save resources.

The two means of 55.8 and 0 representing cell survival for the M120 and O120 respectively, seem to clearly indicate that the M120 cell survival was greater. This observation was also justified with a formal hypothesis test based on the sample statistics obtained from the surviving fraction results:
Table 4. Sample statistics for M120 and O120

<table>
<thead>
<tr>
<th></th>
<th>M120</th>
<th>O120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Survival</td>
<td>0.82</td>
<td>0</td>
</tr>
<tr>
<td>Sample Size</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.0242</td>
<td>0</td>
</tr>
</tbody>
</table>

The t-test result was 82, which is within the critical region of 2.353 with 95% degree of confidence [25]. There was sufficient evidence to support the claim that the M120 mean cell survival is greater than the O120 mean cell survival.

The above graph also shows the plating efficiency of the cells (O). The established plating efficiency for the V-79 hamster lung cells is 70%, which is what the cells used in the experiment also produced. The mean cell survival calculated to be 68.3 per 100 with a standard deviation of 0.58.
CONCLUSION

Based on data collected, the use of SiP on V-79 Chinese hamster cells conveys some degree of radioresistance. Additional studies with larger sample sizes would be needed in further research since this was a small scope study due to the amount of available resources. These experiments will contribute data for the V-79 Chinese hamster lung cell line and results observed from testing the SiP molecule as a radioprotector. As we saw from the data previously presented, the SiP molecule made a difference in the number of surviving cells at the 60 rad level, and made a very significant difference at the 120 rad level.

The final goal of this research is to be able to inject a molecule into patients before radiation therapy that will protect normal cells from radiation damage and reduce the number of normal cells that would otherwise die from the radiation exposure, and reducing symptoms of radiation sickness.
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APPENDICES
APPENDIX A.1
Irradiator Dose Chart

APPENDIX
OREGON STATE UNIVERSITY
RADIATION CENTER
GAMMACELL 220
CHAMBER CROSS SECTION
ISODOSE CURVE

REGION:
A = 7.952E+04 R h⁻¹
B = 8.412E+04 R h⁻¹
C = 8.973E+04 R h⁻¹
D = 9.534E+04 R h⁻¹
E = 1.099E+05 R h⁻¹
F = 1.066E+05 R h⁻¹
G = 1.122E+05 R h⁻¹
H = 1.178E+05 R h⁻¹
I = 1.234E+05 R h⁻¹
J = 1.280E+05 R h⁻¹
K = 1.348E+05 R h⁻¹

Revised 2/00
THE GAMMACELL 220 is the most popular of the AECL line of high dose rate research irradiators. Over 200 have been installed in laboratories throughout the world. They are being used in a wide range of studies in the fields of medical product sterilization, biological and genetic effects, food preservation, growth stimulation, chemistry, pollution, radiation effects on materials, sterile male technique for insect control, and the irradiation of semi-conductors.

Features of the Gammacell 220

- Self shielded — can be placed right in the laboratory. No further shielding is necessary.
- High dose rate — up to 2.0 x 10^6 Roentgens/hour when loaded with 24,000 curies of Cobalt 60. Dose rate and loading can be suited to customer requirements.
- Safe — source permanently fixed in shielded body. No possible malfunction can expose the operator to the source.
- Simple to operate — no special training required.
- Reliable — simplicity of design combined with AECL's long experience in irradiator design and construction assure years of trouble-free operation.
- Large radiation chamber — 15.2 cm (6.0 in) diameter x 20.8 cm (8.12 in) high. Volume: 3738 cm³ (226 in³). The chamber is located in a vertical drawer which automatically positions the chamber in the centre of the radiation field.

THE GAMMACELL 220 is...
APPENDIX A.3
Cobalt-60 Irradiator Specifications

Safety Features
For the protection of the operator several safety features have been incorporated in the unit.

Three microswitches are mounted on the collar door to ensure that:
A) the sample chamber door is properly located.
B) the locking ring is in position.
C) both collar doors are closed.

A fourth microswitch is located on the top shielding plug to ensure that the plug is closed. Unless all four microswitches are actuated the drive motor will not start.

A solenoid operated ram, mounted on the underside of the head, actuates when the drawer stops in the raised position. In this position the ram prevents the drawer from falling should any part of the drive mechanism fail.

A solenoid operated door interlock ensures the collar doors can only be opened with the drawer in the safe position.

Top plug rest and safety column ensure the top plug can only be opened with the drawer in the full up position.
APPENDIX B
Contents of McCoy's 5A Medium

INORGANIC SALTS
- Calcium chloride anhydrous
- Potassium chloride
- Magnesium sulfate anhydrous
- Sodium chloride
- Sodium bicarbonate
- Sodium phosphate monobasic monohydrate

VITAMINS
- Ascorbic acid
- Biotin
- D-Calcium pantothenate
- Choline chloride
- Folic acid
- Myo-inositol
- Niacin
- Niacinamide
- PABA
- Pyridoxal HCl
- Pyridoxine HCl
- Riboflavin
- Thiamine HCl
- Cyanocobalamin

AMINO ACIDS
- L-Alanine
- L-Arginine HCl
- L-Asparagine monohydrate
- L-Aspartic acid
- L-Cysteine HCl monohydrate
- L-Glutamic acid
- L-Glutamine
- Glycine
- L-Histidine HCl monohydrate