Evaluation of Radiation Therapy Produced Cherenkov Light Emissions Used for Photo-activation of Psoralen (AMT)

by

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Justus Adamson

Thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in the Graduate Program in
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2022

ABSTRACT

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Abstract

Purpose: Radiotherapy Enhanced by Cherenkov photo-Activation (RECA) is a novel radiation treatment method that seeks an anti-cancer effect with the introduction of a psoralen compound administered for treatment. The goal of the RECA method is to enhance standard radiation therapy treatments with the addition of psoralen being photo-activated by Cherenkov radiation that is generated during radiotherapy. The purpose of this work is to investigate the effectiveness of RECA on 4T1 mCherry FLuc breast cancer cells seeded on a psoralen-baked-agarose-based rat brain slice.

Methods: A previously established CellProfiler pipeline, developed in our lab by Holden *et al.*, was used to assess tumor burden on rat brain slices used for a tissue-equivalent medium for cell culturing. The CellProfiler pipeline was implemented on images of 4T1 breast cancer cells growing over the course of four to five days post-treatment to measure the average intensity of fluorescing cells.

Prior to the RECA experiment, multiple preparatory experiments were conducted to refine and optimize experimental techniques. The first preparatory experiment tested the possibility of a plate reader bias effect, i.e., signal from nearby wells contributing to signal of other wells, seen during measurements of cell luminescence within individual wells of a clear-bottom 96-well plate. A CellTiter-Glo

endpoint readout was taken 48-hours post-treatment for an endpoint measure to assess the if there was any added signal from nearby wells in the clear-bottom plates.

The next experiment tested whether fractionation of dose was feasible and preferrable to single dose treatment by irradiating 4T1 mCherry Fluc cells with 2 Gy and 4 Gy of kV radiation with and without fractionation. An endpoint CellTiter-Glo readout was conducted 72 hours post-treatment to assess cell viability between the treatment plans.

Additional preparatory experiments investigated whether psoralen-doped agarose was an effective method for cell loading. A 30 μM AMT-baked agar base was placed in half of the wells in plates with 4T1 mCherry Fluc cells seeded on brain slices on top of the agar. One plate received no treatment and one plate received treatment of 365 nm UVA, and an endpoint Firefly Luciferase reporter assay was conducted 48 hours post-treatment to assess cell viability between the conditions.

For the RECA experiment, five 12-well plates, each containing 1 cm of agar with a 400 μm thick coronal slice of rat brain tissue, were given one of five conditions of treatment: no treatment, 4.95 Gy of fractionated kV or MV treatment, or 4.95 Gy of whole kV of MV treatment. Each plate condition consisted of six wells containing AMT-baked agar and six wells containing a standard agar base. After irradiation, images were taken of each of the plates for each day over the course of five days five days with a

Zeiss Lumar microscope. The microscope was equipped with a rhodamine filter to analyze the luminescence readings from each well for assessment of cell viability.

Results: The preparatory experiments all yielded results that allowed for development of the RECA experiment procedure. Investigation of the plate reader effect showed that background signal from nearby wells was not leaking into well signal readout, with all wells having nearly consistent signal throughout all the wells. Fractionating the dose was found to be preferable because it decreased cell viability less than delivering all dose at once, which floored cell viability. Testing psoralen-doped agar demonstrated that this is an effective delivery method for psoralen to intercalate with cells.

The RECA experiment utilizing kV and MV whole dose conditions allowed comparison between irradiations with and without a fractionation scheme. The Firefly Luciferase reporter assay signal for the MV treatment conditions showed less cell viability than the Dark control conditions for both AMT and DMSO. Additionally, the whole dose MV conditions demonstrated a more pronounced decrease in cell viability than the fractionated MV conditions, as expected. The CellProfiler analysis demonstrated the same trends with the whole dose MV AMT condition (8.54 ± 0.99-fold increase) and whole dose MV DMSO condition (11.80 ± 0.70-fold increase) demonstrating less cell viability than the Dark AMT (13.41 ± 0.83-fold increase) and Dark DMSO (14.11 ± 0.62-fold increase). Interestingly, there was not a significant difference in cell viability seen between the fractionated and whole dose conditions.

Conclusions: The procedural techniques developed for the analysis of the RECA effect during the preparatory experiments ruled out a plate reader effect and demonstrated that introducing fractionation and psoralen-baked agar is effective. The testing of the fractionation scheme used for kV irradiations proved to be sufficient for decreasing cell viability without killing all the cells. Additionally, the testing of the psoralen-baked agarose slabs proved to be an adequate psoralen delivery method when compared to methods that used cells suspended in psoralen treated media in prior studies. When these changes to the procedure were introduced together during MV irradiations, the RECA effect did not clearly replicate the results demonstrated during kV irradiations in the preparatory experiments. Further investigation is required to confirm and validate the RECA effect generated during radiotherapy.

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1. Introduction

1.1 Psoralen Derivatives & Cytotoxicity

Psoralen is a natural compound that is extracted from various plants and can be utilized for treatment of skin and extracorporeal diseases/disorders such as psoriasis, eczema, and vitiligo, when combined with ultraviolet light. This compound can be taken either orally or applied topically with the patient submerged in a psoralen bath. Psoralen demonstrates a strong wavelength dependence in which it is allowed to be photo-activated by ultraviolet light. Photo-activation typically occurs when an incident photon with a specific energy and wavelength, which is predominantly UVA, interacts with the psoralen causing it to be photo-activated. UVA photons have a depth of penetration of about 1 mm, limiting treatment to superficial diseases for psoralen to be adequately photo-activated.

When photo-activation occurs, psoralen molecules form covalent bonds to the 5′-tpA-3′ sites within the DNA. The first photo-activation of psoralen causes a 4′, 5′ monoadduct to be formed. The second photo-activation causes a double bond to form between the thymine base and 3, 4 sites, which then form a crosslink. The resultant monoadducts and crosslinks from the photo-activation of psoralen generate immunogenetic effects including the promotion of development, maturation, and proliferation of T-cells, up-regulation of immunogenic transcription factors, and up-regulation of major histocompatibility complex I.

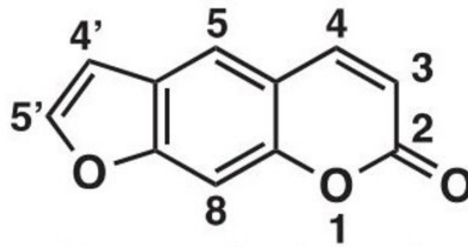

Figure 1: Psoralen Numbering Scheme

These are known systemic immunogenic effects for superficial treatment involving psoralen. This shows promise for treatment of non-superficial tumors if psoralen can be photo-activated at the site of non-superficial tumors.

1.2 Cherenkov Phenomenon

Cherenkov radiation is electromagnetic radiation given off when a charged particle, when travelling through a dielectric medium, is moving faster than the phase velocity of light in the dielectric medium. Cherenkov radiation is inherently generated during external beam radiation therapy (EBRT) when treating patients with LINACs.

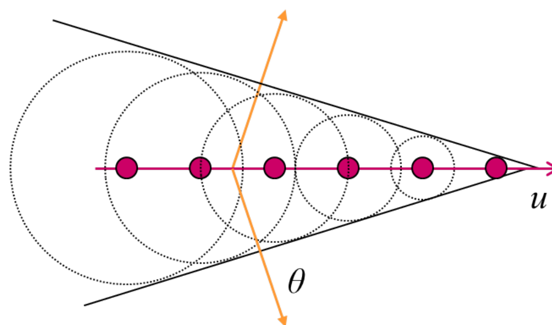

Figure 2: Depiction of Cherenkov radiation radially emitted from a charged particle with velocity u at an angle θ

Cherenkov radiation demonstrates a linear relationship with the dose used during irradiations, meaning that the emission of Cherenkov is independent to dose rate so long as the total dose being given remains constant.

Additionally, Cherenkov light emission occurs at a maximum wavelength of 420 nm. These emissions fall within the range of UVA light, which is between 315 nm and 400 nm, thus this range of wavelength is optimal for photo-activation of psoralen compounds.

1.3 Prior Work With RECA

Preliminary investigation leading into the RECA experiments began with an analysis of the fluence of Cherenkov light emissions. Shrock *et al.* investigated the fluence of Cherenkov light emissions using Monte Carlo simulations consisting of varying filters, beam energies, and dose rates. These parameters were evaluated to see their effects on beam fluence. Ultimately, using a higher beam energy and low dose rate, when accompanied with an aluminum filter, generated the most Cherenkov light per unit of dose used during treatment. An investigation into the proper wavelength for psoralen photo-activation was conducted to determine the appropriate range of light required for efficient psoralen photo-activation. It was found that there is a peak crosslinking region in which wavelengths within the UVA range are optimal for psoralen photo-activation.

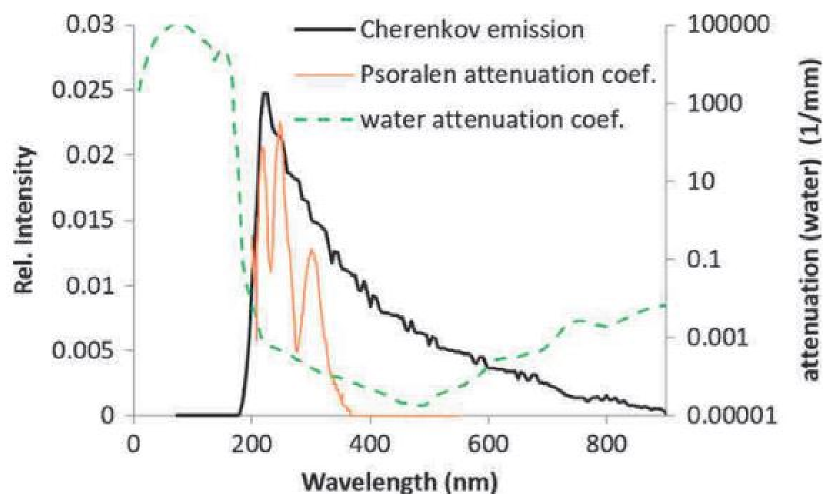

Figure 3: Plot comparison of Cherenkov light emission spectrum to psoralen attenuation spectrum.

Within this region, there is a close relationship between Cherenkov emissions and psoralen absorbance around 300 to 320 nm. Given that psoralen absorbance occurs between the wavelength range of 200 nm to 400 nm, this crosslinking region indicates that Cherenkov light emissions from a LINAC are adequate in photo-activating psoralen compounds.

Another study conducted by Yoon *et al.* was done to investigate the RECA effect when coupled with radiotherapy in comparison to standard radiotherapy treatments. This was done to assess the effect on cytotoxicity when a RECA component was added to the radiotherapy treatment of two cancer cell lines: B16 melanoma and 4T1 breast cancer cells. During treatment, plated cells were placed on top of 3 cm of solid water and irradiated with a 6 MV PA beam to generate Cherenkov light from the block of solid water beneath the plates of cells. Cells irradiated with 6 Gy of dose expressed a 250%

increase in MHC I expression and cells irradiated with 4 Gy of dose expressed a 450% increase in MHC I expression. MHC I expression is important in terms of production of proteins for the immune system, thus an increased MHC I expression indicates an increased immune response. Additionally, B16 and 4T1 cells receiving 2 Gy of dose from a 6 MV PA beam demonstrated a 9.5% and 20% increased in cytotoxicity, respectively. Overall, these results show promise that Cherenkov light is adequate in photo-activating psoralen as well as having an increased immunogenic effect.

Building upon work published by Yoon *et al.*, another study conducted by Sagarika *et al.* sought to conduct a quantitative analysis of the Cherenkov emissions given off by x-ray megavoltage (15 MV) irradiated materials including solid water, animal tissues, and india ink doped agarose gels. This experiment used a low noise charge coupled camera for low light measurements of the Cherenkov light generated during radiotherapy. The camera was placed in a black, light-blocking chamber on the LINAC couch at an angle to minimize stray UV light with the addition of 10 cm lead bricks to minimize background radiation. Additionally, a band-pass filter was used to eliminate other light spectra such as UVB, visible light, and infrared light. It was found that solid water generated 66 ± 5%, 64 ± 5%, and 76 ± 3% less UVA than chicken breast, pork loin, and pork belly, respectively. When compared to chicken breast, 250 ppm india ink doped agarose gel generated within 8% of the same amount of Cherenkov light. This suggests that 250 ppm india ink doped agarose gel is adequate in mimicking Cherenkov

emissions during radiotherapy, as chicken breast is often used as a tissue-equivalent reference.

Lastly, a study conducted by Holden *et al.* investigated the RECA method by irradiating 4T1 mCherry Fluc cells treated with AMT psoralen with MV or kV radiation treatments. An analysis of the integrated intensity measured through CellProfiler showed a decrease in tumor proliferation after the course of five days. The MV control condition demonstrated 5.65 ± 0.78-fold growth and the MV condition treated with AMT demonstrated a 3.49 ± 0.52-fold growth. This result suggests that the psoralen treatment with AMT influences the radiotherapeutic effect on cells, causing a decrease in cell proliferation. Additionally, the kV control and kV AMT conditions demonstrated a slightly smaller effect on cell proliferation, which was a 6.73 ± 1.24-fold and 5.26 ± 0.59-fold growth, respectively. Lastly, the cell proliferation in the dark control condition that received no AMT treatment or radiation had a growth that was on par with the 13.6 ± 1.5 hour doubling time associated with 4T1 breast cancer cells.

1.4 Rat Brain Slice In-Vitro Technique

The standard procedure followed for early RECA experiments used standard well plates for in-vitro culturing of cancer cells. However, a limitation to this culturing technique may not be considered "tissue-equivalent' and is much more difficult to replicate the growth of cells in the human body. Additionally, this also limits the ability

to replicate accurate Cherenkov light emissions that would occur during standard radiotherapy treatments on human tissue.

Starting from the study conducted by Holden *et al.,* a technique of utilizing a novel Rat Brain Slice culturing model was implemented into the RECA experiments. The model involves using a rat brain slice of a few hundred micrometers in thickness to culture the cancer cells on rather than using a plastic well base with added media.

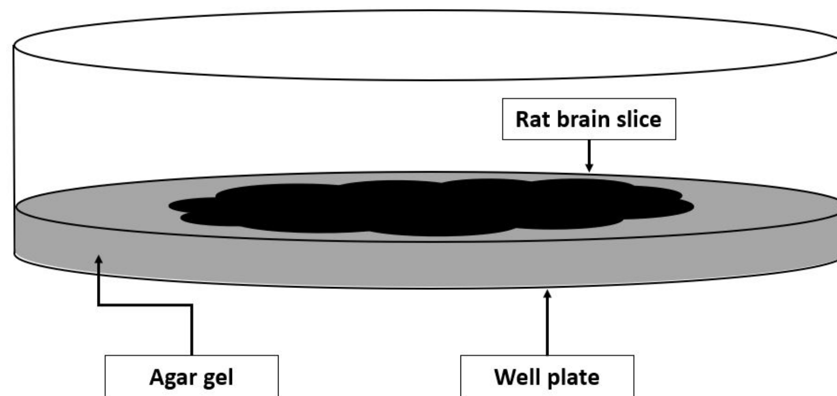

Figure 4: Well setup using cells seeded on rat brain slice resting on agar gel molded within the wells of plates.

The purpose of this change is to mimic the cellular interactions in a tissue model as well as better replicate Cherenkov light emissions that would occur in the presence on tissue. This helps simulate a scenario closer to in-vivo rather than limiting the experiment to strictly in-vitro.

1.5 CellProfiler Image Analysis

CellProfiler is an openly available and free software that is designed to perform quantitative analysis of phenotypes from images provided by users. A CellProfiler

pipeline called the Fluorescent Cell Burden (FCB) method was developed during a study by Holden *et al.* to quantitatively measure the luminescence of cells given off by 4T1 mCherry Fluc cells imaged under a Zeiss Lumar Microscope. FCB uses CellProfiler to perform analysis on the images to provide an outline of what is believed to be considered a cancer cell colony based on the luminescence given off by the cells. This is done by adjusting parameters such as object diameter range, block size, and intensity range.

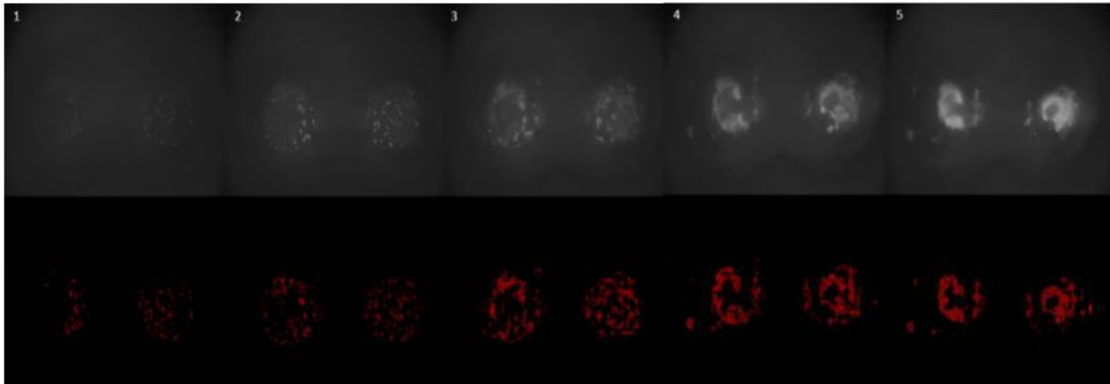

Figure 5: Example of CellProfiler analysis. Grayscale images of 4T1 mCherry signal over five days (top), and cell colony outlines from CellProfiler (bottom).

The efficacy and accuracy of the FCB method was tested by Holden *et al.* by generating a series of simulated images through MATLAB with known luminescence values given throughout the image. The FCB method used on 10 simulated images measured an integrated intensity across all images with an accuracy of 99.23% ± 0.75%. Additionally, when cancer cell colony density was increased, it was found that the FCB method began to overestimate the measured integrated intensity, but was still within 5% of the known, simulated values. Overall, the FCB method using CellProfiler has been

tested and shown to be accurate in outlining cancer cell colonies based on intensity of image luminescence.

1.6 X-PACT

A study conducted by Oldham *et al.* uses a method called X-ray Psoralen Activated Cancer Therapy (X-PACT), which shows promise with using psoralen in the treatment of non-extracorporeal cancers. X-PACT uses psoralen with phosphors that can be injected into the tumor directly, adding a psoralen treatment component to standard radiotherapy. The cancerous area receives kV X-ray treatment to activate the injected phosphors. The activation of the phosphors yields lower energy UVA photons after the incident X-ray is absorbed, which is then used to photo-activate the psoralen that is also present within the tumor.

An in-vivo study was conducted on syngeneic 4T1-HER2 tumors grown on BAB/c mice. The tumors were treated with UVADEX (8-MOP) psoralen in combination with phosphor and subjected to 1 Gy of dose from 80 kVp X-rays. As a result, 4T1-HER2 tumors receiving a combination of psoralen and phosphor treatment with the treatment of X-rays showed a drastic increase in cytotoxicity when compared to tumors without the psoralen and phosphor treatment component. Additionally, an in-vitro study was conducted on the syngeneic 4T1-HER2 tumors in BALB/c mice with tumors being treated with X-rays and a combination of X-rays with psoralen and phosphor. BALB/c mice receiving X-ray treatment in conjunction of psoralen and phosphor demonstrated a

slower 4T1-HER2 tumor growth. Both results suggest that the addition of a psoralen treatment component, when photo-activated, demonstrates an increased therapeutic effect when compared to X-ray treatment without the addition of psoralen and phosphor.

1.7 Project Scope

The results of previous RECA studies have shown potential for an enhanced radiotherapeutic effect with the addition of psoralen. RECA was developed to combat limitations present in the X-PACT study to continue investigation of using psoralen during radiotherapy. Mainly, RECA sought to utilize Cherenkov emissions generated during radiotherapy using a LINAC to photo-activate psoralen treated cancer cells. Although RECA was developed to address limitations present during the X-PACT study, the RECA method still has limitations. To overcome these limitations, this work seeks to refine the RECA experimental procedure in testing this treatment method in terms of the amount of dose delivered, dose fractionation, psoralen delivery methods, and psoralen loading times, as well as quantify the RECA effect achieved through photo-activation of AMT using Cherenkov light.

2. Preparatory Experiments

This thesis research consists of several complicated and intricate experiments in which procedures were developed over the course of multiple years and studies. The RECA experiments contain many variables that contribute to the outcome of the experiment; thus, it is important to ensure that sufficient work is done to develop an effective procedure. For this reason, it imperative that several tests and experiments are conducted in preparation for the RECA experiments. One of the most important components in this work is the psoralen drug compound being used to demonstrate RECA.

2.1 AMT Psoralen Compound

Psoralen derivatives have an inherent dark cytotoxicity associated with them and express a different amount of cytotoxicity when photo-activated using ultraviolet light. Psoralen cytotoxicity measurements were compared by Buhimschi *et al.* to see the cytotoxicity expression between different psoralen derivatives. 4'-Aminomethyltrioxsalen (AMT) was found to be a psoralen derivative that expresses a limited amount of cytotoxicity in the absence of light and expresses a higher cytotoxicity with the addition of ultraviolet light photo-activation. These cytotoxicity characteristics are important when considering the impact on cancer treatment since we only want to see an anti-cancer effect when psoralen is photo-activated by ultraviolet light and not see cell death caused by addition of psoralen alone. Additionally, it is imperative to ensure

that AMT is an adequate choice for psoralen drug treatment by requiring adequate efficacy, short loading times, limited ambient light activation, limited dark cytotoxicity, and is photo-activated at various doses during irradiations.

2.1.1 How effective is the AMT psoralen derivative?

Initial investigation into the efficacy of AMT as a psoralen derivative began with comparing AMT to 8-Methoxypsoralen (8-MOP) in terms of cytotoxicity when treating 4T1 mCherry Fluc cells with 365 nm UVA. This was done to assess the AMT psoralen derivative as an alternative to 8-MOP, which was used in previous studies, but it was reported that 8-MOP was less potent than AMT by Buhimschi *et al.* To assess these differences, 1 μM of either AMT to 8-MOP was given to 4T1 mCherry Fluc cells cultured in well plates 1 hour prior to UVA treatment. The cells were treated with 0.25, 0.5, or 1.0 J/cm^2 of 365 nm UVA irradiations to photo-activate the psoralen derivatives. An endpoint CellTiter-Glo and Firefly Luciferase assay measurement was done on all wells to assess cell viability after UVA treatments. AMT was validated as demonstrating more potency than 8-MOP. The cells treated with 1 μM of AMT treated with 365 nm UVA expressed significantly less cell viability post-treatment when compared to the control condition with only Dimethyl Sulfoxide (DMSO) present, and less cell viability than the 1 μM 8-MOP condition. This validates the choice of using AMT as a psoralen compound, in terms of cytotoxicity with photo-activation and psoralen potency, for future studies.

2.1.2 Is AMT photo-activated by ambient light?

Psoralen derivatives generally display some amount of cytotoxicity when photo-activated by light, mainly in the wavelength range of ultraviolet light; however, psoralen

derivatives can express cytotoxicity with partial photo-activation from ambient light or stray light from background. To test the ambient light activation of AMT, two conditions were compared: UVA light treatment and ambient light treatment. For the ambient light treatment, a red-light filter was placed over the lights present within the lab when plating and treating cells. This was done to ensure that any light reaching the AMT-treated cells would not photo-activate the psoralen, causing it to be cytotoxic prior to irradiations. 4T1 cells for this condition were exposed to 10 minutes of red filtered light. The UVA light condition exposed 4T1 cells to 10 minutes of 0.5 J/cm^2 365 nm UVA. An endpoint CellTiter-Glo measurement was made for each of the conditions to compare cell viability post-treatment. It was found that cells treated with AMT in the presence of UVA exhibited much less cell viability than cells receiving UVA treatment without AMT present. Additionally, when compared to the ambient light condition, cell viability was unchanged, suggesting that ambient light was not photo-activating the AMT and causing it to become cytotoxic prior to irradiations.

2.1.3 Is AMT cytotoxic without photo-activation?

As stated previously, psoralen compounds express inherent cytotoxicity in the absence of photo-activation from outside sources. To test the dark cytotoxicity of AMT, 4T1 mCherry FLuc cells were cultured in wells with varying concentrations of AMT. The plated cells were not subjected to any radiation treatment and were shielded from any background light by keeping the plate covered.

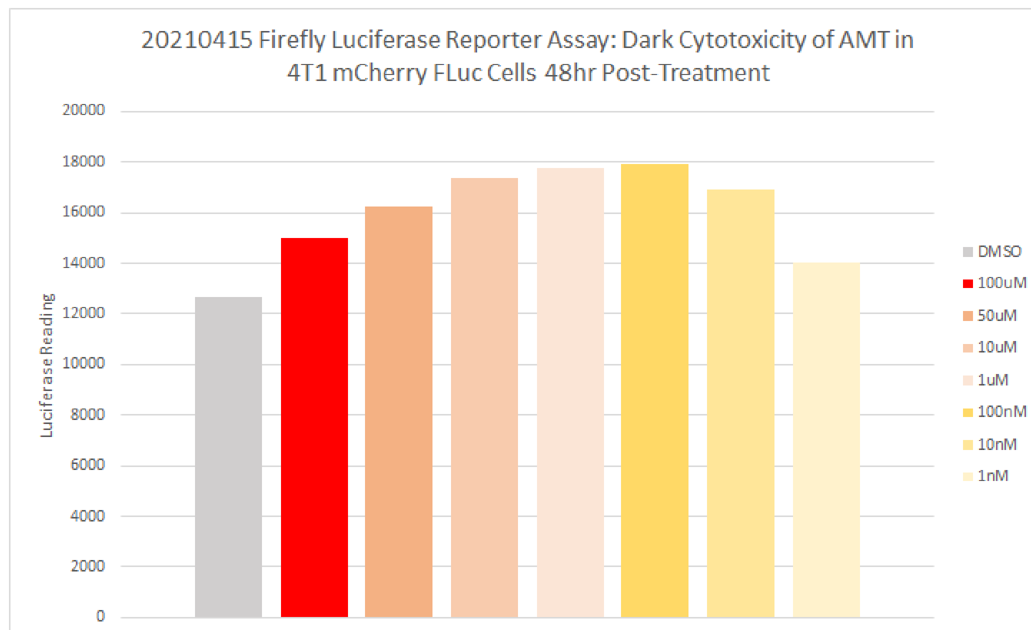

Figure 6: Average Firefly Luciferase assay signal 48 hours post-treatment compared between 1 nM and 100 µM of AMT with a DMSO control without AMT.

An endpoint Firefly Luciferase assay reading was done 48 hours after cells were treated with AMT. A strong trend to suggest dark cytotoxicity was not seen throughout the changing concentrations of AMT in the absence of light. It is worth noting that a strong trend is still not seen in the 100 µM AMT condition, which is a 10^2 increase from the concentration used in previous RECA experiments.

2.1.4 What dose & concentration of AMT is most effective?

A change in radiation dose used for irradiations was brought up after concerns of cell viability being decreased to much by the radiation treatment alone. This would make the enhanced radiotherapeutic effect from RECA harder to point out. Additionally, investigation of psoralen loading times began after a literature search

revealed that many psoralen studies use loading times that are longer than an hour, which is the amount of time used for previous RECA experiments. This is done to increase the potency of psoralen to the cells. To investigate dose titration and psoralen loading times, an experiment was conducted to irradiate plates of 4T1 mCherry Fluc cells with either 2 Gy or 4 Gy of MV irradiation. Additionally, each dose condition had two loading time conditions of either one hour of incubation or three hours of incubation.

Results show that the 2 Gy was not as harsh on the tissue and cancer cells when compared to a higher dose of 4 Gy, as expected. Cell viability for plates that received 4 Gy was decreased much more than the cell viability for plates that received only 2 Gy. Additionally, when comparing psoralen loading times, results suggest that allowing the 4T1 cells to incubate for a longer time after being treated with AMT ensured that the AMT was able to better intercalate with the cells. This suggests that using a lower dose given to the cells at any given time is preferred as well as extending the psoralen loading time to give the AMT a better chance of fully intercalating with the cells.

2.2 Plate Reader Bias Effect

Investigation into a plate reader effect was conducted after seeing a trend in decreased cell viability towards the middle of 96-well clear-bottom plates used in previous experiments. This study consisted of plating about 5,000 4T1 mCherry Fluc cells into four 96-well plates: two opaque-bottom plates and two clear-bottom plates.

Plates with the same bottom type received two different plating schemes in which varying wells were filled with AMT and cells. In addition to these conditions, the concentration of AMT within the wells varied from 10 nM to 100 μM. The four plate conditions followed the set up as shown in the figure below.

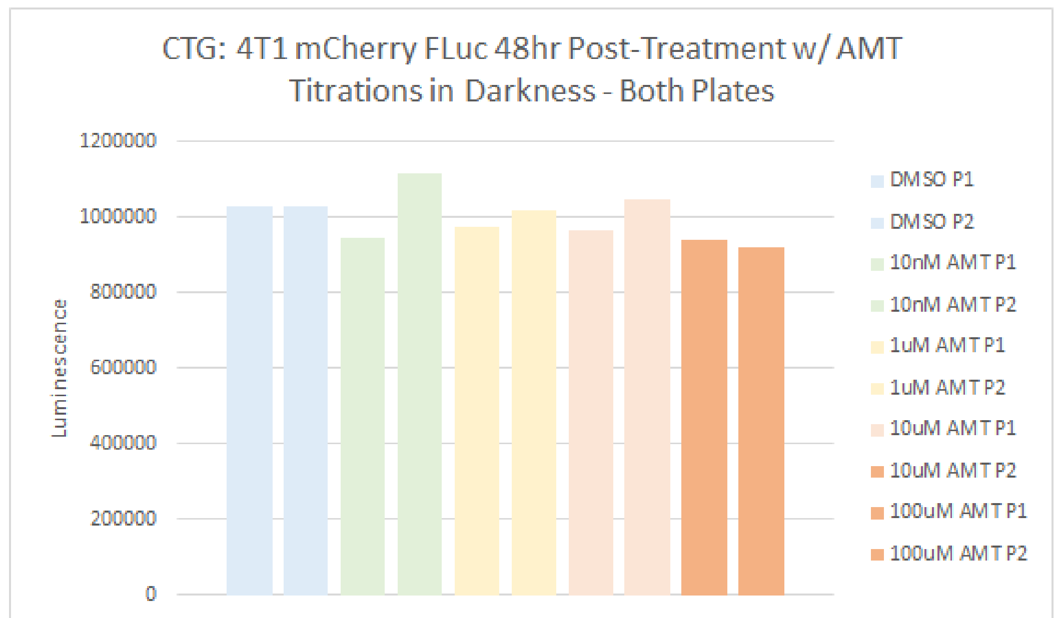

Figure 7: Comparison of CellTiter-Glo average luminescence of cells from varying doses of AMT in a clear-bottom plate (P1) and opaque-bottom plate (P2)

The plates did not receive any light or radiation treatment and an endpoint CellTiter-Glo measurement was made 48 hours post-treatment. this was done to see the impact that the type of plate bottom would have on the readout values. The CellTiter-Glo analysis showed that a plate reader effect was not seen in either type of plate or was a plate reader effect seen using the different plating schemes. This was indicated by consistent readings throughout the wells around each of the plates.

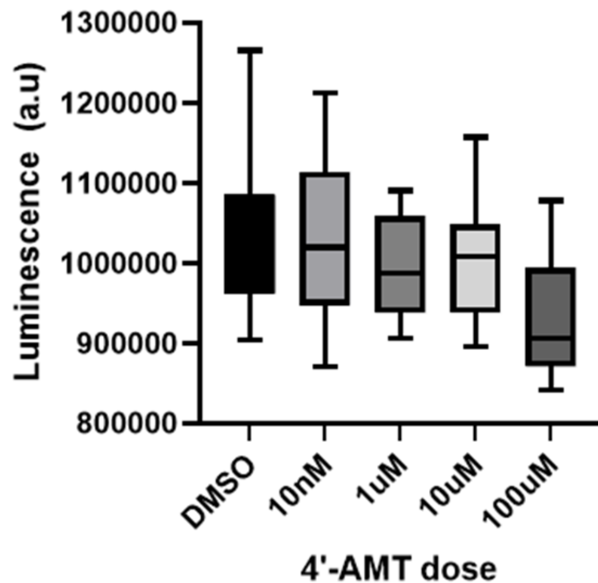

Figure 8: Average CellTiter-Glo luminescence measurements between varying doses of AMT to corroborate to changing cell viability.

Additionally, it was found that the concentration of psoralen has a significant impact on cell viability without the addition of light or radiation. This suggests that the higher values towards the middle of the plate in previous experiments were attributed to the wells having higher concentrations of AMT present and not to due with the type of plate used. It was found that the 100 μM AMT concentration had the highest decrease in cell viability, suggesting that this concentration of AMT exhibits some cytotoxicity to the cells even in the absence of light and radiation.

2.3 Fractionated UVA Dose Validation

A proof-of-concept validation experiment was carried out to test the introduction of fractionation schemes for the kV and MV irradiations given to the 4T1 mCherry Fluc cells. To test the concept of fractionating the dose, this experiment tested four plate

conditions which either received all prescribed dose at once or received the same prescribed dose over multiple fractions. An XRAD 160 Irradiator was used to deliver kV irradiations to all four plates. The first plate received 46 seconds of kV irradiation to deliver an equivalent 2 Gy of dose and a second plate received 92 seconds of kV irradiation to deliver an equivalent 4 Gy of dose. A third plate received 2 Gy fractioned into six fractions of about 0.33 Gy for eight seconds and a fourth plate received 4 Gy divided into six fractions of about 0.67 Gy for sixteen seconds with each fraction occurring six hours apart over three days. An endpoint CellTiter-Glo readout was done to assess cell viability 72 hours post-treatment.

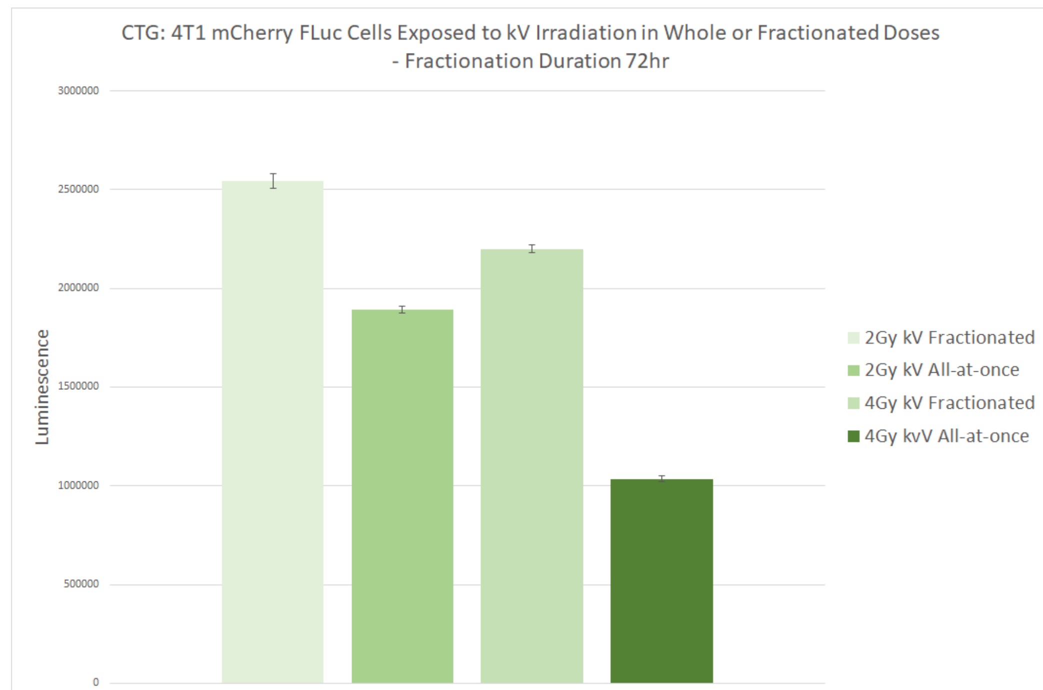

Figure 9: CellTiter-Glo readout of luminescence values 72 hours post-treatment. Comparison of 2 Gy and 4 Gy doses all-at-once to 2 Gy and 4 Gy fractionated doses.

As seen in the figure above, the fractionation of dose demonstrates an effect on the cells that is not as harsh on the cancer cells. The plates receiving a total dose all at once demonstrate lower total luminescence of cells in the plates, which shows that cell viability is decreased the most when the dose is delivered at one time. The fractionated plates demonstrate cell viability that is higher than the whole dose plates, suggesting that using a fractionation scheme is preferred when looking for indications of RECA in future experiments.

2.4 Psoralen-doped Agar Delivery

Inspired by previous neuroprotective compound experiments conducted by Denise Dunn, an experiment was created to test the feasibility of using psoralen-treated agarose gel as a drug delivery method to the cells cultured on brain slices placed on top of the agar. This experiment used two 12-clear-well plates to compare six wells of 30 μM AMT-treated agar to six wells of standard 0.6% DMSO agar without psoralen treatment. 4T1 mCherry Fluc cells were seeded on brain slices resting on brain slices, which were placed on one of the two agar conditions. One of the 12-clear-well plates was treated with 0.5 J/cm^2 of 365 nm UVA and the other plate did not receive any UVA treatment. An endpoint Firefly Luciferase assay readout was done 72 hours post-treatment to assess the cell viability in both plates.

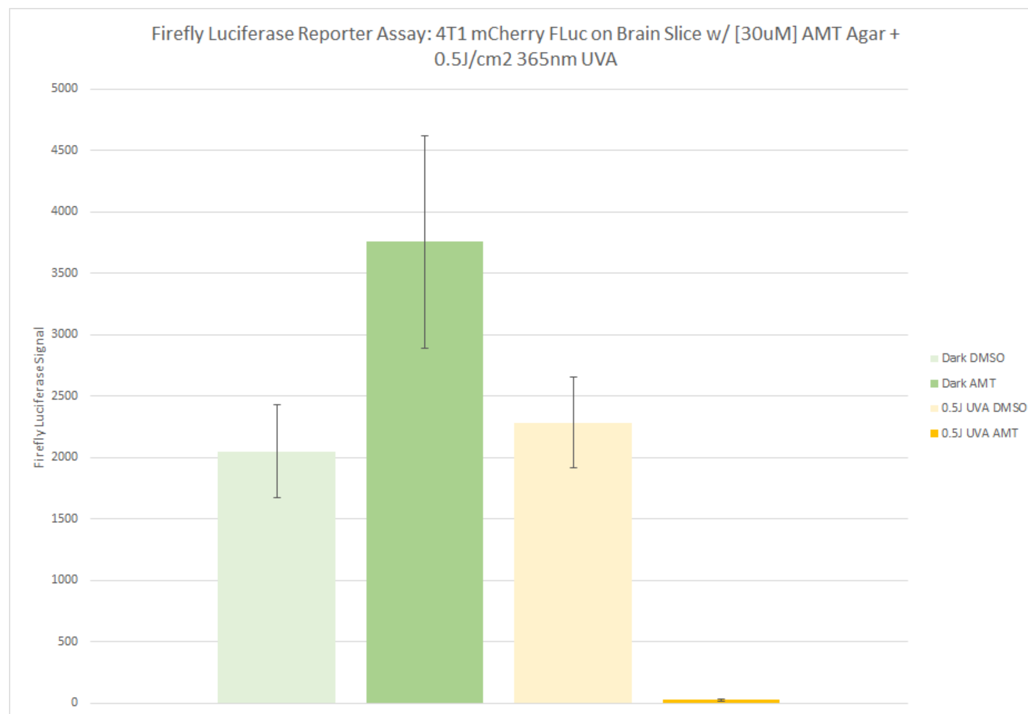

Figure 10: Average endpoint Firefly Luciferase assay signal of dark conditions compared to UVA treatment with and without AMT.

The 72-hour post-treatment readout confirmed that the psoralen-baked agar is a viable psoralen delivery method for the brain slices. The wells containing AMT-treated agar without UVA treatment did not demonstrate a cytotoxic effect from the AMT itself; however, the AMT-treated agar, when combined with UVA treatment, drastically decreased cell viability. This indicates that the psoralen-treated agar is effectively delivering the psoralen to the brain slices and intercalating with the 4T1 cells.

A similar experiment was conducted using the same procedure as before with the incorporation of 30 μM 8-MOP-treated agar to compare psoralen cytotoxicity. Additionally, a fractionated dose condition was added for comparison of the whole dose

and fractionation dose conditions. The fractionation scheme involved three, timed fractions of 0.5 J/cm² 365 nm UVA over the course of three days to achieve an equivalent dose of the whole dose conditions.

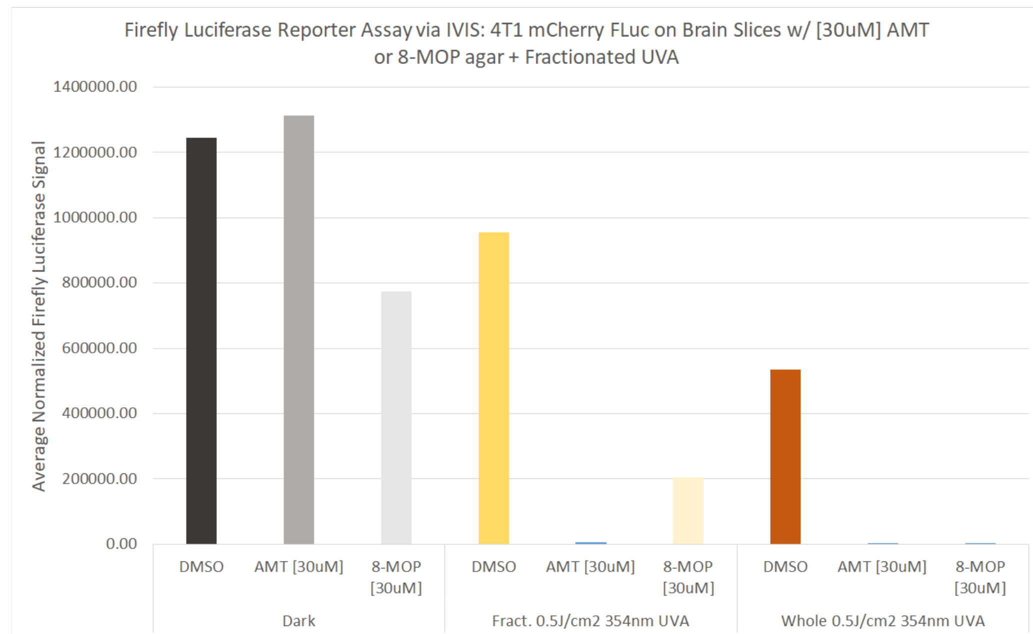

Figure 11: Average Firefly Luciferase assay signals from DMSO, AMT, and 8-MOP. Comparison of signals between dark, fractionated UVA, and whole UVA treatments.

Like the prior experiment, the psoralen-baked agar demonstrated that it is a viable psoralen delivery method. Additionally, it was seen that AMT was slightly less cytotoxic to the cells in the dark conditions when compared to 8-MOP. Lastly, the fractionation of UVA dose proved to be less harsh on the cells that did not receive a psoralen treatment, meaning that this allows for a better visual when analyzing the effect that psoralen has on treatment of the cells when combined with a UVA treatment.

3. Methods: Main RECA Experiment

3.1 Agarose Preparation

5 mg of AMT powder were procured and dissolved in 3,400 μL of DMSO to create a 5 mM stock solution. This was used to create the AMT-baked agar to be placed in 30 of the 60 total wells. A 0.5% agar block was prepared to mimic a tissue phantom for Cherenkov emissions, which will be placed beneath the plates in the whole-dose and fractionated MV irradiations. 900 mL of heated water was added to 4.5 g of agarose, which is molded to be 2.5 cm high in a glass flat-bottomed 8x8 inch baking pan. Note that the 2.5 cm agar height was chosen because 0.5 cm of agar is already present within each of the wells. The agar is then cooled to solid form in a 4°C cold room for about four hours.

3.2 RECA Experiment Procedure

Five 12-well agar plates are procured with one 350-micron hemispheric rat brain slices in each well atop of 1 mL of 0.5% solid media agar. There two different types of agar preparations that are created. A 0.6% DMSO control agar was created by adding 198 μL of DMSO to 32.802 mL of media and agarose mixture. A 30 μM AMT agar was created by adding 198 μL of 5mM AMT stock added to 32.802 mL of media and agarose mixture.

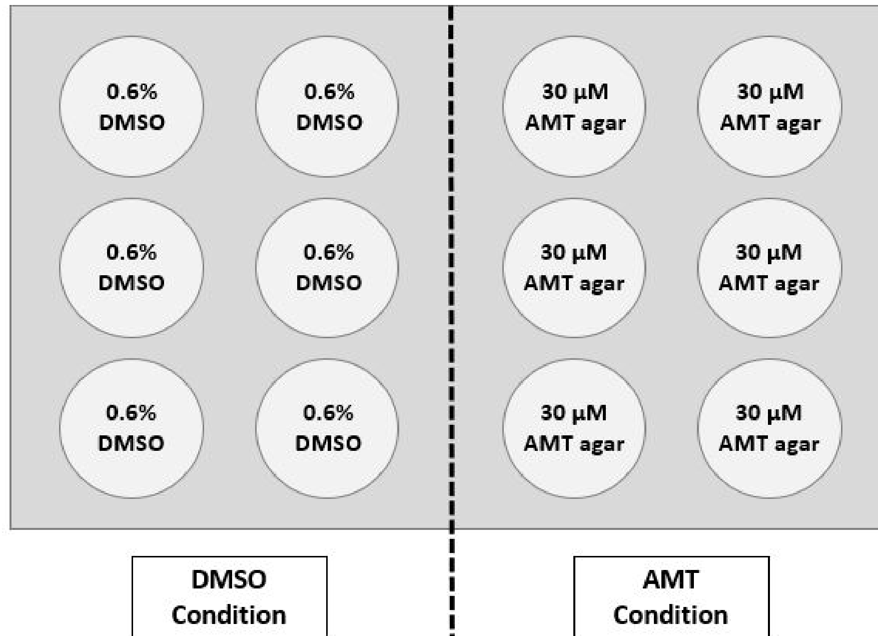

Figure 12: Layout of wells for 12-well plate used for main RECA experiment.

3.2.1 MV Irradiation and Imaging

Immediately upon procurement of the slice plates, 10,000 4T1 mCherry FLuc cells in 2 μL aliquots are seeded on brain slices in each well. Plates are then returned to a 37°C 5% CO2 incubator for 24 hours to allow the AMT within the agar to intercalate with the DNA of the 4T1 cells. After the 24 hours of incubation, pictures are taken with the Zeiss Lumar stereoscope with an added rhodamine filter using a 1500.0 ms exposure time. The first image is taken for a pre-treatment measure of cell viability. All plates are transported together to Duke Hospital in a sealed Styrofoam box with warming bags to minimize variation from location and temperature.

Table 1: Breakdown of imaging times using Zeiss Lumar Microscope

	Day 0	Day 1	Day 2	Day 3	Day 4
Time elapsed	Immediate	24 hours	48 hours	72 hours	96 hours

Plates are centered in a 20x20 cm light field atop of the 2.5 cm agar block resting on the LINAC couch at 100 cm SSD. MV plates receive either the first of three fractions of 1.65 Gy or 4.95 Gy for a whole dose from the 15 MV LINAC with a 180° gantry rotation. The total dose was originally set at 4.5 Gy, but it was increased to 4.95 Gy total to account for an estimated 10% of dose from backscatter losses. Additionally, a dose rate of 600 MU/min was used for MV irradiation.

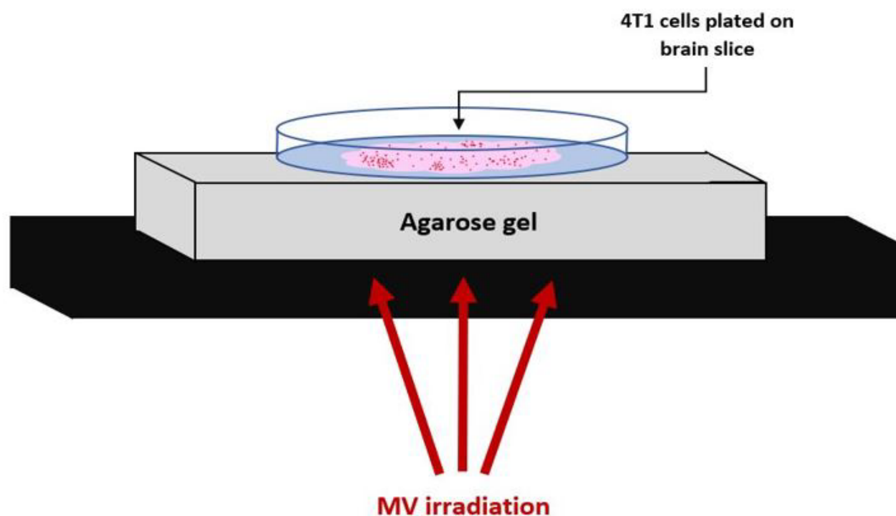

Figure 13: Schematic of 15 MV irradiation setup of 4T1 cells seeded on living tissue on top of AMT agar. Plate is resting on an agarose gel.

After MV irradiations, all plates are transported back to the lab for kV irradiations of the kV plates using a XRAD 160 irradiator that uses 0.5 J/cm² 365 nm UVA. kV irradiation were included as a comparison to MV irradiations, as kV radiation

and MV radiation demonstrate a radio-biological effectiveness (RBE), which causes kV irradiation to cause more DNA damage during treatment. An equivalent dose of 4.95 Gy is delivered to one plate over 113 seconds and the first fraction of 1.65 Gy over 38 seconds delivered to the fractionated plate. After kV irradiation, all plates are transported and returned to the incubator to allow the 4T1 cells to culture for another 24 hours. This procedure is repeated for two more days with images taken before each irradiation for the fractionated MV and kV plates.

24 hours after the third and final fraction of dose, the last set of stereoscope images of the brain slices are taken. After imaging, measurements are obtained from the Firefly Luciferase report assay signal.

Table 2: Treatment conditions delivered to 4T1 cells during main RECA experiment.

Plate Condition	Radiation Type	Dose [Gy]	Fractionated?
Control	-	No dose given	-
MV Whole Dose	MV	4.95	No
MV Fractionated	MV	4.95	Yes (1.65 Gy x 3)
kV Whole Dose	kV	4.95	No
kV Fractionated	kV	4.95	Yes (1.65 Gy x 3)

After the endpoint Firefly Luciferase assay signals are obtained, the brain slices from each well are saved for further investigation and future experiments. For each plate condition, slices are aggregated into two separate 1.5 mL tubes and flash-frozen in liquid nitrogen. These samples are stored at -80°C for future use.

3.3 CellProfiler Image Analysis

Following irradiation and endpoint Firefly Luciferase report assay, images of each brain slice over each day were gathered for image analysis using a CellProfiler pipeline. A standard greyscale image of the firefly luciferase reporter assay signal was passed through the CellProfiler pipeline to create an outline of what is believed to be cancer cell colonies present in the images.

Figure 14: Original greyscale image of the brain slice (left), image of fluorescing cells with rhodamine filter (middle), CellProfiler outline image (right).

Parameters were adjusted to ensure that the outline of the cancer cell colonies was accurate enough such that objects within the image were being outlined correctly and background signal or auto-luminescence from the brain slice were not being outlined by the pipeline. To do this, a block size of 30 pixels was used as the size for analyzing areas of the image. An approximate object diameter of 15 pixels was assigned as the diameter of objects that the pipeline was analyzing. Additionally, the lower and

upper bounds of primary objects that the pipeline was to identify was 0.015 and 1.0 pixels, respectively.

4. Results

Results for this research consisted of two separate analyses of cell viability: Firefly Luciferase reporter assay and CellProfiler image analysis. The Firefly Luciferase reporter assay analysis used luminescence signal given off by 4T1 mCherry Fluc cells that give off a signal when an endpoint readout is performed. This correlates directly with cell viability as living 4T1 cells treated with the assay are the only source of signal to be measured. Additionally, the CellProfiler image analysis consisted of analyzing images of the brain slices over the course of four to five days. An image was taken of each brain slice over each day to analyze the luminescence signal given of by the cells when imaged with a rhodamine filter attached to the Zeiss Lumar microscope. The CellProfiler pipeline outlines cell colonies based on the signal given off by the cells in the images, which also correlates directly to cell viability.

4.1 Firefly Luciferase Reporter Assay

A Firefly Luciferase reporter assay endpoint measurement was conducted on the 4T1 mCherry FLuc cells that received either no treatment, fractionated kV irradiation, fractionated MV irradiation, whole kV irradiation, or whole MV irradiation. The endpoint firefly luciferase signal is shown in Figure 17 below.

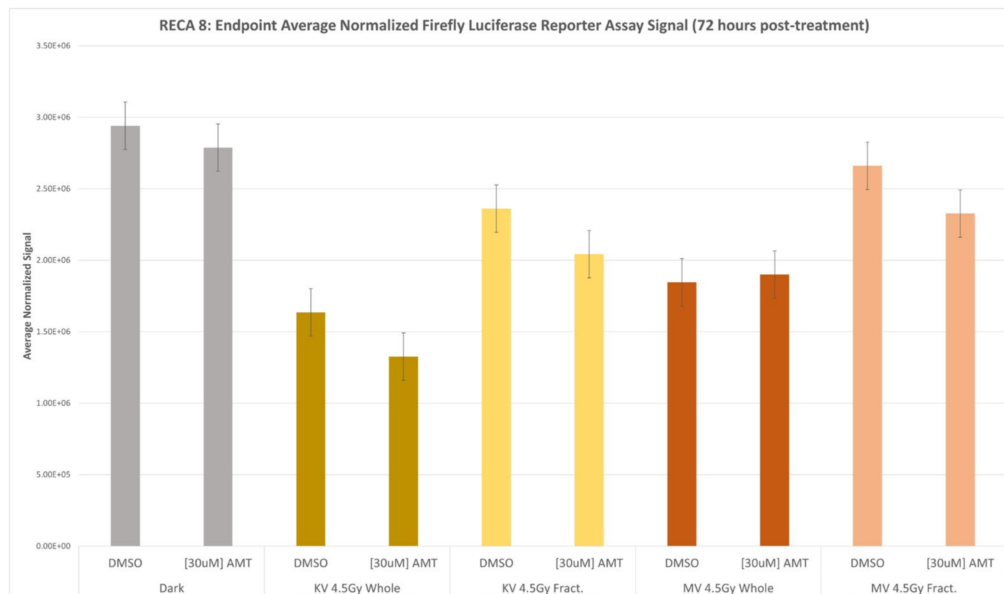

Figure 15: Average normalized signal from Firefly Luciferase reporter assay after 72 hours to compare plate conditions.

These results from this RECA experiment demonstrated slight improvement in signal throughout all conditions when compared to preliminary results gathered from our first attempt at the RECA experiment. Both the fractionated and whole MV conditions showed lower cell viability when compared to the dark control condition, as expected. Additionally, the fractionated conditions demonstrated higher cell viability when compared to their whole dose counterparts, which was also as expected. Cell viability from the conditions that received kV treatment demonstrated the lowest cell viability, which was lower than the conditions that received MV treatment. This can be attributed to the difference in RBE between radiation types.

4.2 CellProfiler Image Analysis

The CellProfiler image analysis was conducted on the Day 1, Day 2, Day 3, and Day 4 images of the brain slices for each condition and treatment type to get an improved analysis of cell growth over the 72 hours. Like the previous experiment, the CellProfiler analysis showed slight improvement in the assessment of cell viability when compared to the Firefly Luciferase reporter assay.

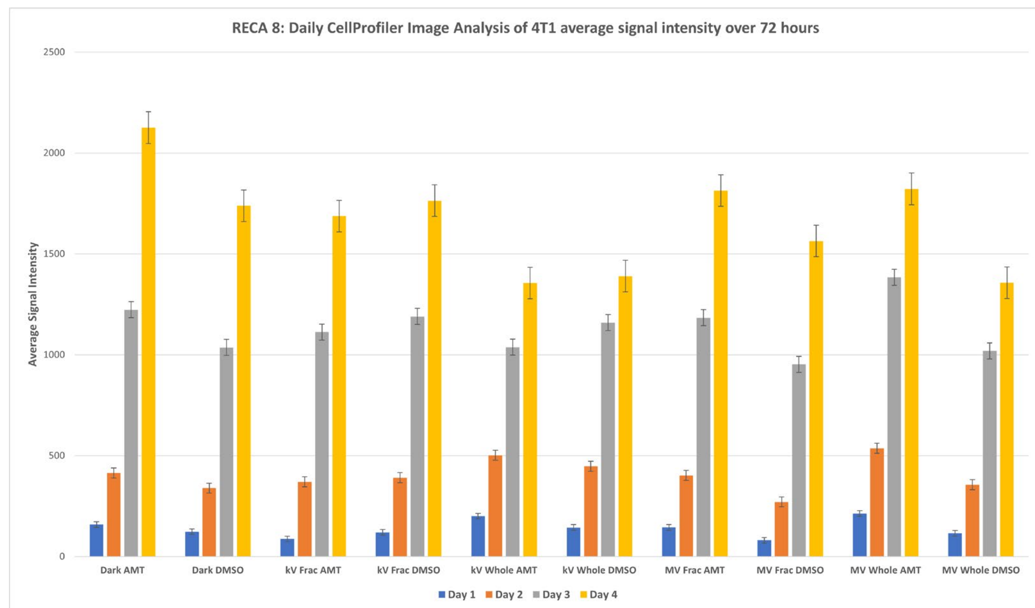

Figure 16: Daily measurements of CellProfiler outlined 4T1 colony average signal intensity to compare plates conditions.

When comparing the average signal intensity from cells outlined by the CellProfiler outline, there was improvement seen in the comparison of cell viability between the dark control conditions and conditions receiving radiation treatment. The AMT and DMSO conditions receiving a whole MV treatment demonstrated an 8.54 ± 0.99-fold increase and 11.80 ± 0.70-fold increase in cell viability, respectively. This is an

improvement when compared to the 13.41 ± 0.83-fold increase and 14.11 ± 0.62-fold increase seen in the dark conditions of AMT and DMSO, respectively. Interestingly, we did not see the difference in cell proliferation between the fractionated and whole dose treatments that we saw with the kV irradiations during preparatory experiments.

5. Discussion

The early results gathered from preparatory experiments are promising and show potential for the procedure developed to investigate the RECA effect. The experiments conducted to investigate the plate reader effect caused by added signals from nearby wells within a plate was ruled out and not seen when using different plate schemes; however, the variation in signal suggests that increased luminescence values could be attributed to the concentration of AMT used for treatment. The experiment investigating the concept of dose fractionation proved that using a fractionation scheme is less harsh on the cells of the living tissue as well as the cancer cells, allowing visualization of the RECA effect if it occurs. This is important as we do not want to floor the cell viability from the irradiation alone. Lastly, the investigation of using psoralen-doped agar proved that the AMT was intercalating with the cells seeded on rat brain slices, further showing that this psoralen delivery method is adequate and works efficiently. This is also important to ensure that the psoralen is properly intercalating with the cells.

When all previous work investigated during preparatory experiments were put together, the RECA effect was not clearly shown in the measurements of firefly luciferase assay signal or luminescence, which are both surrogates for cell viability. This was shown by the 8.40 ± 0.98-fold growth seen in the MV AMT condition and 12.22 ± 0.79-fold growth seen in the MV AMT fractionated condition when compared to the MV

control that showed a 13.08 ± 0.83-fold growth. Although there is slight improvement in which the 4T1 cells are growing, there was still significant growth of the 4T1 cells over the course of 72 to 96 hours. This was unexpected as we did not replicate the results observed in preparatory experiments. Although extensive work has been done to refine the RECA experimental procedure, further investigation of these results and a repeat of this experiment could aid in explaining these results as well as potentially show a more defined RECA effect.

6. Conclusions

Current experimental setup and procedures were constructed from a series of preparatory studies. The study testing the implementation of fractionation schemes used for kV irradiations proved to be sufficient for decreasing cell viability without completely killing all the cells. Additionally, the experiment that tested the delivery method of psoralen-doped agarose slabs proved to be adequate in allowing psoralen to intercalate with cells when compared to cells that were suspended in psoralen treated media.

The results from the main RECA experiment did not clearly suggest that a RECA effect was being seen from radiotherapy treatments. Further effort in making changes to the experimental setup and procedure is needed to continue investigation of the RECA effect.

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