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Amifostine Prophylaxis in Irradiated Breast Reconstruction: A Study of Oncologic Safety *In Vitro*

Alexandra O. Luby, MS[1,*], **Chitra Subramanian, PhD**[2,*], **Lauren K. Buchman**[1], **Jeremy V. Lynn, BS**[1], **Kevin M. Urlaub, BS**[1], **Noah S. Nelson, MPH**[1], **Alexis Donneys, MD**[1], **Mark S. Cohen, MD**[2], **Steven R. Buchman, MD**[1]

[1]University of Michigan Health Systems, Section of Plastic Surgery, Department of Surgery, 1540 E. Hospital Drive, Ann Arbor, MI, USA

[2]University of Michigan Health Systems, Section of General Surgery, Department of Surgery, 1500 E. Medical Center Drive, Ann Arbor, MI, USA

Abstract

Background—Indications for adjuvant radiation therapy (XRT) in breast cancer have expanded. Although highly effective, XRT damages surrounding tissues and vasculature, often resulting in delayed or compromised breast reconstruction. Thus, effective, yet safe methods of radiation injury prophylaxis would be desirable. Amifostine is a FDA-approved radio-protectant, however, concerns about its potential to also protect cancer remain. The purpose of this study was to evaluate the oncologic safety of Amifostine *in vitro* and determine its effect on human breast cancer cells in the setting of XRT.

Methods—One ER+/PR+/Her2- (MCF-7) and two ER-/PR-/Her2- (MDA-MB-231,MDA-MB-468) breast cancer cell lines were investigated. Female Fibroblasts (FF) were utilized as controls. Cells were treated with WR-1065, the active metabolite of Amifostine, 20 minutes before 0Gy, 10Gy, or 20Gy XRT. Live and dead cells were quantified; percent cell death was calculated.

Results—WR-1065 treatment significantly preserved viability and reduced healthy FF death after XRT compared to untreated controls. All three breast cancer cells lines exhibited radio-sensitivity with substantial cell death. Cancer cells retained their radio-sensitivity despite WR-1065 pretreatment, achieving the same degree of cell death as untreated controls.

Conclusions—This study demonstrated the proficiency of Amifostine to selectively protect healthy cells from XRT, while breast cancer cells continued to remain radiosensitive. These results support the oncologic safety of Amifostine in breast cancer *in vitro.* Further investigation is now

Pre-publication corresponding author: Alexandra O. **Luby**, MS, alexalub@med.umich.edu, Phone: (330) 322-0591. Post-publication corresponding author: Steven R. **Buchman**, MD, 1540 E Hospital Drive, SPC 4219, Ann Arbor, MI 48109-4219, sbuchman@med.umich.edu, Phone: (734) 763-8063, Fax: (734) 232-6973.

*Denotes Equal Contribution Authorship

Author Contributions

Alexandra Luby and Chitra Subramanian were the primary authors of this manuscript, designed and performed all experiments, and performed data acquisition and analysis. Lauren Buchman assisted with all experimentation and data collection. Jeremy Lynn, Kevin Urlaub, Noah Nelson, Alexis Donneys, and Mark Cohen also assisted with the experimentation, data analysis, microscopic images, and supplemented information to this manuscript. Steven Buchman was the principal investigator of this project and made edits to multiple drafts of this manuscript.

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warranted *in vivo* to ascertain the translational potential of using Amifostine as a radio-protectant for breast reconstruction after radiation treatment.

Introduction

Breast cancer is the most common cancer diagnosed in the United States, with approximately 253,000 new cases of invasive breast cancer diagnosed in 2017.[1,2] Amidst new screening protocols, earlier detection, and improved treatment options, the majority of patients diagnosed with breast cancer are treated with curative intent.[3] As such, breast cancer survivors are often forced to live with the aftermath of treatment, which is a reality faced by a growing number of breast cancer survivors, estimated to be approximately 3.1 million women in the United States alone.[4] While surgical resection remains the primary treatment for breast cancer; there are increasing indications for adjuvant XRT after breast conserving surgery and post-mastectomy,[5–7] as it is a highly effective treatment shown to reduce disease recurrence.[8]

Despite advancements in radiotherapy, acute and long term sequelae associated with XRT persist after treatment concludes.[9–11] Radiotherapy has profoundly destructive short and long-term effects on skin, soft tissue, and surrounding vasculature. XRT-associated pathologic injury to healthy tissue not only prolongs patients' road to recovery, but also poses major obstacles to achieving a timely and satisfactory breast reconstruction outcome.[12–14] Autologous breast reconstruction is often delayed until months after radiotherapy completion, while implant-based approaches bear higher rates of complications and failure in the setting of XRT, thereby limiting the reconstructive options available to both breast cancer patients and their surgeons. Plastic and reconstructive surgeons continue to work towards solutions to overcome the unique clinical challenges faced in the aftermath of radiation therapy.

Additionally, in certain cases, some XRT associated sequelae are so severe and poorly tolerated that they limit the dose of radiation patients can receive, which can compromise local tumor control and significantly impact oncologic treatment outcomes.[10,15] Considering that healthy tissue tolerance limits radiotherapy dosing, there is increasing clinical utility for radio-protective agents that selectively reduce XRT-induced injury in healthy tissue without decreasing the tumoricidal efficacy of XRT.[16,17] Such agents would not only reduce insult to healthy tissue and improve reconstructive options available to patients and plastic surgeons, but would also allow for higher doses of XRT to be administered and better tolerated by cancer patients.

Currently, no therapeutics exist to prevent radiation injury in breast cancer patients. Previous studies in our laboratory have identified Amifostine (AMF) as a potential therapeutic solution to this unaddressed clinical problem. While AMF is one of only a few FDA approved prophylactic radio-protectants, indicated to prevent xerostomia in patients with head and neck cancer receiving radiotherapy, it has not been investigated for use in breast cancer patients receiving XRT. One major barrier to utilizing AMF as a radio-protectant in breast cancer patients is remaining uncertainty whether AMF may potentially protect breast cancer cells from XRT in addition to protecting healthy cells. While the oncologic safety of

AMF in the setting of head and neck cancer has been well studied,[18–20] it has yet to be investigated in the setting of breast cancer. Therefore, the objective of this study was to evaluate the oncologic safety of Amifostine and determine its effect on human breast cancer cells in the setting of XRT. Our hypothesis is that Amifostine will afford radio-protection to healthy cells, but will not extend protection to breast cancer cells. Determining the oncologic safety of AMF in breast cancer *in vitro* is a critical preliminary step in ascertaining the translational potential of utilizing AMF as a radioprotectant in breast cancer and breast reconstruction patients receiving radiation therapy.

Methods

Study Cell Line Selection

Validated triple negative (ER-/PR-/Her2-) breast cancer cell lines, MDA-MB-231 and MDA-MB-468, and ER+/PR+/Her2- breast cancer cell line, MCF-7, were investigated (Table 1). Female Fibroblasts (FF) served as a healthy control cell line in this study. All cells were grown in two-dimensional culture in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic Antimycotic in a humidified $C0_2$ incubator.

Standardization Study to Determine Experimental Conditions

To identify appropriate doses of XRT and AMF, breast cancer cell lines were treated with varying doses of XRT (0, 5, 10, 20 Gy) and WR-1065 (0, 0.125, 0.25, 0.5, 1.0, 2.5, 5.0 mM). Each experimental condition was evaluated in triplicate. Cells grown to 70% confluence were trypsinized and counted using hemocytometer. Approximately 25,000 MDA-MB-231, MDA-MB-468, and MCF-7 cells and 50,000 FF cells were suspended in 450 μl of media and seeded in 24-well plates. 24 hours following plating, the cells were treated with the above concentrations of WR-1065, the active metabolite of AMF. Cells were exposed to WR-1065 for 20 minutes, then washed and the media was replaced. Immediately following pharmacologic treatment, the cells underwent XRT. After 48 hours, trypan blue counting was utilized to calculate cell viability of each treatment group. Based on this dose response, the concentration of WR-1065 and the dosing of XRT was selected for the evaluation of cancer cell response at radio-protective treatment conditions.

Evaluating the Effect of Radiation Therapy after Amifostine Pretreatment

The cells were plated as previously described. 24 hours after plating, the cells were treated with a final concentration of 0.25 mM WR-1065 for 20 minutes, while untreated cells served as a control. Following this exposure, cells were washed with sterile PBS and fresh media was added. Immediately thereafter, a single dose of 0, 10, or 20 Gy XRT was administered. Breast cancer cell response to treatment was evaluated 48 hours following XRT.

Prior to analysis, images of the cells were captured utilizing light microscopy. Then, the media from each well containing the detached dead cells was collected. Wells were washed with 50 μl of PBS, which was then collected. 200 μl of trypsin was added to the remaining cells attached to the plate. Cells were incubated at 37°C with trypsin for 3 minutes, and subsequently neutralized with 400 μl of media with FBS. The volume isolated from each of

these steps (media removal, wash, neutralization) was collected in a microcentrifuge tube for each well. The isolate was then evaluated utilizing a trypan blue staining assay. Live and dead cells were quantified.

Data Analysis

Average values for each well were calculated based on three sample counts. Overall average for each cell line and treatment condition was then calculated based on the average value of each well. Percent cell death and percent cell viability was calculated. Two sample t-test was performed to compare values between groups and two-tail p-values were determined, where $p<0.05$ was taken to be significant.

Results

Breast Cancer Cells Remain Radio-Sensitive after WR-1065 Treatment

Both triple negative cancer cell lines, MDA-MB-468 and MDA-MB-231, exhibited radio-sensitivity with substantial cell death at 10 and 20 Gy (Table 2). In fact, cell death did not differ between triple negative breast cancer cells, MDA-MB-468 and MDA-MB-231, pretreated with WR-1065 and their untreated controls at 10 Gy and 20 Gy (Figures 1–2). Additionally, the ER+/PR+/Her2- breast cancer cell line, MCF-7, also demonstrated radio-sensitivity at both 10 Gy and 20 Gy (Figure 3). Cell death did not differ between MCF-7 pretreated with WR-1065 and the untreated control. Also, of note, higher levels of cell death were seen at 20 Gy for all three breast cancer cells lines as expected (Figure 2).

WR-1065 Mitigates Cell Death in Female Fibroblasts Receiving XRT

WR-1065 treatment of fibroblasts significantly preserved FF cell viability and conversely reduced healthy FF cell death after XRT compared to untreated controls at 10 Gy and 20 Gy. At 10 Gy, untreated fibroblasts exhibited 39.3% cell death 48 hours after irradiation, while fibroblasts pre-treated with WR-1065 experienced 14.5% cell death ($p<0.001$). Additionally, at 20 Gy, the cell death of untreated fibroblasts was 52.7%, whereas WR-1065 pre-treated fibroblasts demonstrated 19.4% cell death ($p=0.008$) (Figure 4). Notably, there was no significant difference in cell death between irradiated fibroblasts receiving radio-protective treatment at 10 Gy and 20 Gy and the non-irradiated controls. Therefore, WR-1065 treatment demonstrated a highly significant radio-protective effect in healthy female fibroblasts at both 10 Gy and 20 Gy.

Microscopic Images Uphold Cell Death Findings

To supplement and support these quantified findings, microscopic images were taken of each of the wells 24 hour after plating, which we denote as t=0, and 48 hours after XRT. As shown, these images demonstrate the marked reduction in the viable number of cells, the change in cell morphology, and the presence of rounded cells, denoting dead or dying cells. There is a marked decrease in the number of living cells on the plate comparing the plates at 48 hours post-XRT. For all three breast cancer cell lines, 0 Gy serves as a baseline, showing marked proliferation, while there is a marked decrease in cell density at 10 Gy, and even further reduction in cells at 20 Gy, regardless of WR-1065 pre-treatment (Figures 1–3). This same extent of reduction in cell number was exhibited by untreated FF cells, but was not

seen for the FF cells pretreated with WR-1065 at 10 Gy and 20 Gy, which demonstrated similar plate densities between irradiated and non-irradiated groups (Figure 4).

Discussion

This study highlights important investigational findings validating the preliminary oncologic safety of AMF in breast cancer *in vitro*. Both ER+/PR+/Her2- (MCF-7) and ER-/PR-/Her2- (MDA-MB-231, MDA-MB-468) breast cancer cells remained radiosensitive despite prophylactic treatment with radio-protective doses of WR-1065, the active metabolite of AMF. In fact, there was no significant difference in cell death between breast cancer cells receiving WR-1065 pretreatment and untreated controls at both 10 Gy and 20 Gy. Therefore, not only do breast cancer cells remain radio-sensitive when treated with WR-1065, but they maintain the same level of radio-sensitivity as the untreated controls. WR-1065 pretreatment of control female fibroblast cells, however, did in fact produce a profoundly protective effect. Prophylactic WR-1065 preserved cell viability of healthy female fibroblasts receiving radiation, as fibroblast viability was increased by 24.8% at 10 Gy and by 33.3% at 20 Gy in cells receiving radio-protective pre-treatment compared to untreated controls. Therefore, WR-1065 afforded protection to the female fibroblast cells but did not offer any protection to the three breast cancer cell lines investigated. The results of this study demonstrated that Amifostine is an effective radio-protective agent that selectively reduces XRT-induced injury in healthy FF cells without decreasing the tumoricidal efficacy of XRT in both ER+/PR+/Her2- and ER-/PR-/Her2- breast cancer.

We selected two triple negative breast cancer cell lines (MDA-MB-231 and MDA-MB-468) to investigate in this study, as 10–20% of breast cancer patients present with this type of breast cancer.[21] Triple negative breast cancers do not express the three most common receptors that many therapeutics target: ER, PR, and Her2. Thus, these patients typically have poor prognoses due to the aggressive nature of this cancer and its lack of response to hormone therapy and many chemotherapeutic agents. XRT is the most common and most effective treatment modality for patients with triple negative breast cancer after surgical resection. As such, this patient population would likely benefit from the use of a radio-protective agent like AMF. Therefore, we found it of utmost clinical importance to evaluate the oncologic safety of utilizing AMF in triple negative breast cancer.

In addition to triple negative breast cancer cell lines, the ER+/PR+/Her2- cell line, MCF-7, was studied to evaluate the effect of AMF in receptor positive breast cancer. This type of breast cancer is often treated with chemotherapy and/or radiotherapy in addition to surgical resection, depending on the staging and extent of disease.[22] Furthermore, unlike majority of the triple negative cancer patients who have mutant P53 expression, MCF-7 breast cancers do not have P53 mutations; however, it is well established that P53 plays a major role in DNA damage due to XRT.[23–25] Therefore, it was important to evaluate the effect of AMF on receptor positive MCF-7 breast cancer in the setting of XRT.

Given the high volume of ongoing research on breast cancer treatment protocols, there are expanding indications for adjuvant radiation therapy in the setting of both breast conserving surgery and mastectomy.[5,6] Although XRT is a highly effective component to breast cancer

treatment shown to improve survival,[26] it has profoundly destructive short and long-term effects on the skin, soft tissue, and surrounding vasculature. This can limit reconstructive options and negatively impact the aesthetic outcome in these patients. XRT can delay or compromise autologous tissue flap reconstruction, as it decimates local tissue vascularity, which is critical to flap success.[27] Immediate autologous breast reconstruction with subsequent radiotherapy can lead to skin and flap atrophy, distortion, fibrosis, and fat necrosis.[6,7,28] While some recent studies have demonstrated success with immediate autologous reconstruction,[29] the aforementioned onerous complications often deter surgeons from this approach, opting for delayed approaches instead, which postpones autologous reconstruction months after the conclusion of radiotherapy.

Radiotherapy also complicates implant-based breast reconstruction, resulting in overall higher failure rates and increased incidence of complications, including infection and capsular contracture.[7,30] While some have found that the use of tissue extracellular matrix formulations reduces the incidence of capsular contracture,[31–33] it does not eliminate this burdensome complication, which requires additional surgical procedures for these patients. Despite the potential complications associated with implant-based reconstruction in the setting of XRT, it still remains the most common reconstructive option pursued by women undergoing postmastectomy radiotherapy.[6] Evidenced by the extensive body of literature on this topic, breast reconstruction in the setting of XRT remains a major clinical challenge, with ongoing debate over best practice techniques and timing of interventions. Regardless of the reconstructive approach, there is extraordinary clinical utility for an effective, yet safe method of radiation injury prophylaxis in the setting of breast reconstruction after breast cancer treatment, as it has the potential to increase reconstructive options available to both breast cancer patients and plastic surgeons.

Previous in vivo studies in our laboratory have investigated the use of AMF in irradiated tissue expander based breast reconstruction in a murine model.[34–37] Animals exposed to XRT developed a fibrotic capsule around the tissue expander, extensive fibrosis of the surrounding local soft tissue, increased collagen disorganization, ulceration of local skin, and a reduction of skin vascularity.[14,34–38] AMF was shown to mitigate many of these pathologic effects in vivo,[34–37] therefore, we sought to evaluate the safety of such an efficacious therapy *in vitro* in order to ascertain the translational potential of our findings to the clinical setting.

Satisfactory breast reconstruction is essential to the physical, psychological, and emotional well-being and recovery of breast cancer patients.[30,39,40] As such, oncologic surgical planning has become increasingly collaborative in recent years, where breast surgeons and plastic surgeons partner to deliver the most optimal results from both oncologic and aesthetic perspectives. With broadening indications for XRT in breast cancer, there is a growing need to minimize the destructive effects of XRT on healthy tissue, without decreasing the tumoricidal efficacy of radiation therapy. While some may posit radio-protective agents are only beneficial from a reconstructive perspective, this rationale is inherently flawed, as the use of radio-protectants like AMF also offers the ability to deliver higher tolerable doses of XRT, which can result in more effective disease control and treatment. This is a highly important consideration for breast cancers requiring radiation to the internal mammary

lymph nodes and thoracic cavity, where healthy tissue toxicity can limit XRT dosing, and ultimately, disease control. Therefore, AMF could offer tremendous clinical utility in lymph node positive disease and cancers that utilize XRT as a primary treatment modality, including triple negative breast cancer, which we investigated in this study.

Limitations and Future Implications

While this study evaluated the oncologic safety of AMF in both receptor positive and receptor negative breast cancer *in vitro*, future studies in our laboratory will evaluate its oncologic safety *in vivo*. Addressing the oncologic safety of AMF in applications other than head and neck cancer, however, is not the only obstacle to AMF use in the clinical setting. Admittedly, the side effect profile of AMF has also hindered its clinical adoption. The current FDA approved formulations of AMF are intravenous and subcutaneous. Administered IV and SC, AMF reaches peak levels within seconds to minutes of administration, which can result in nausea, vomiting, and dizziness in a subset of patients. Our laboratory however, has been developing and investigating oral formulations of AMF to reduce peak drug exposure levels and time to peak levels, to ultimately mitigate these side effects.[41] By developing improved methods for AMF administration, reducing its side effect profile, and addressing the oncologic safety of AMF, it is our hope to increase the clinical feasibility of AMF, and therefore, its clinical utility. If AMF is found to be safe and efficacious in shielding the skin, soft tissue, and vasculature of the breast from radiation induced injury, further studies can then be performed to ascertain the translational potential of utilizing AMF as a radio-protectant in breast cancer patients receiving radiation therapy.

Conclusion

Based on the results of this *in vitro* study, prophylactic treatment with AMF mitigates healthy cell death after XRT, but does not impact the tumoricidal efficacy of XRT on breast cancer cells. Radio-protective agents, like Amifostine, provide major advantages in the setting of breast cancer from both oncologic and reconstructive perspectives. AMF offers the ability to deliver higher doses of XRT, which can result in more effective disease control and treatment and improve breast reconstructive outcomes and quality of life for breast cancer survivors. Therefore, AMF is particularly important to consider and evaluate in cancers that utilize XRT as a primary treatment modality, including triple negative breast cancer, which we investigated in this study. While AMF is FDA approved for prophylaxis of XRT in the setting of head and neck cancer, we make the case, based on the results of this study and previous work in our laboratory, that AMF offers tremendous potential clinical utility in the setting of breast cancer and reconstruction. This study demonstrated the proficiency of Amifostine to selectively protect healthy fibroblast cells from XRT, while breast cancer cells remained radiosensitive. These results support the oncologic safety of Amifostine in breast cancer *in vitro*, however, further investigation is now warranted to validate these findings in breast cancer tumors *in vivo*.

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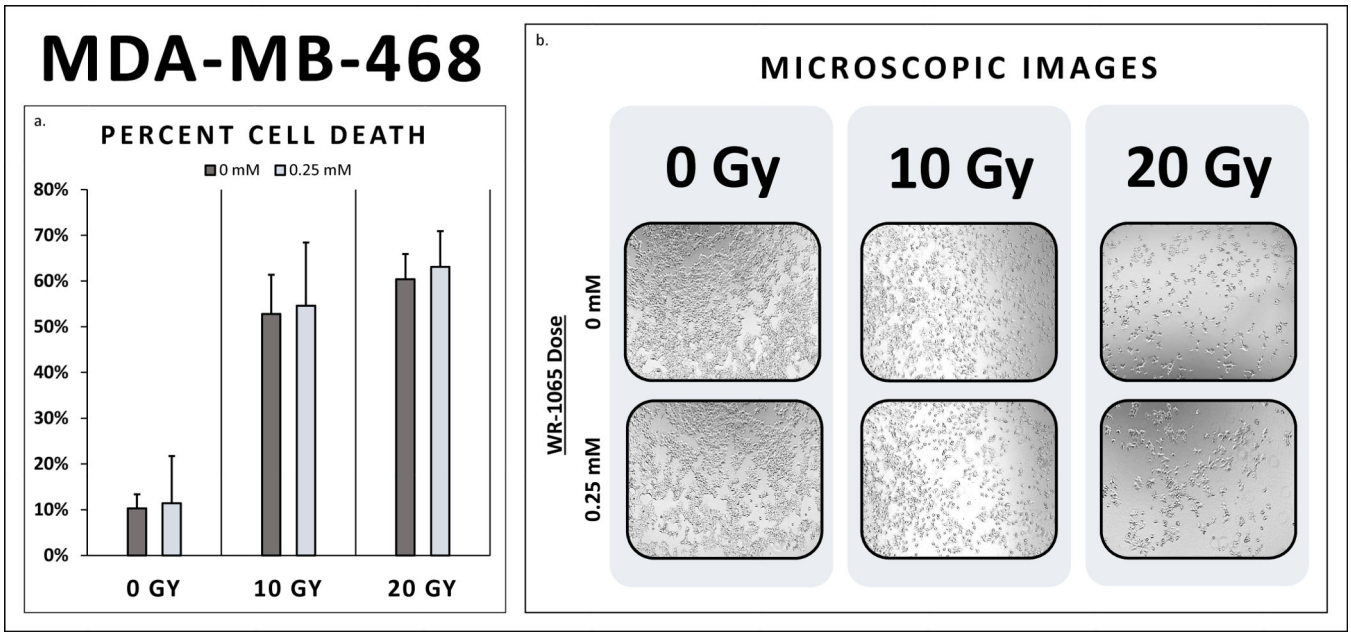

Figure 1:

1a. MDA-MB-468 Percent Cell Death. Cell death did not differ between MDA-MB-468 breast cancer cells pretreated with WR-1065 and their untreated controls at 10 Gy and 20 Gy. Breast cancer cells receiving radio-protective pretreatment reached the same degree of cell death as untreated breast cancer cells. (10 Gy: 54.6% vs. 52.8%, $p>0.05$; 20 Gy: 63.1% vs. 60.4%, $p>0.05$) 1b. MDA-MB-468 Microscopic Images. There was decreased breast cancer cell density at 10 Gy compared to 0 Gy. There was even further reduction in cells at 20 Gy compared to 10 Gy. No remarkable difference in cell density was observed between breast cancer cells pre-treated with WR-1065 compared to the controls at 0 Gy, 10 Gy, and 20 Gy, respectively.

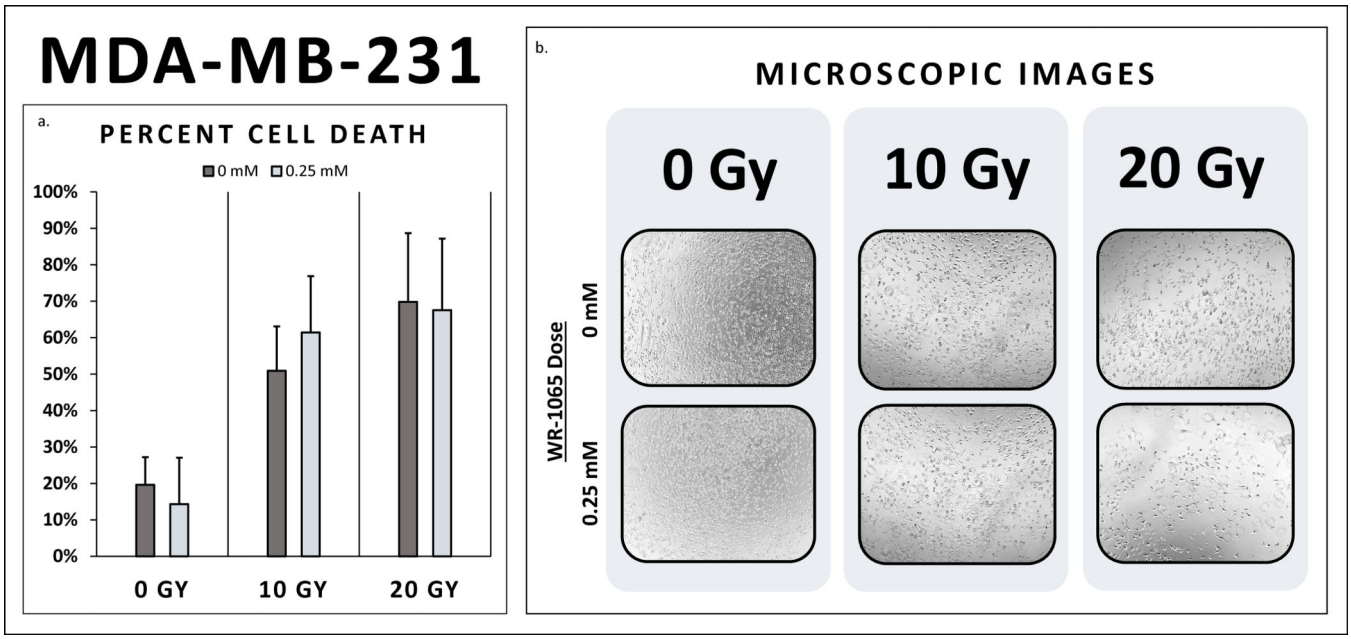

Figure 2:

2a. MDA-MB-231 Percent Cell Death. Cell death did not differ between MDA-MB-231 breast cancer cells pretreated with WR-1065 and their untreated controls at 10 Gy and 20 Gy. Breast cancer cells pretreated with radio-protective doses of WR-1065 achieved the same degree of cell death as untreated breast cancer cells. (10 Gy: 61.4% vs. 50.9%, $p>0.05$; 20 Gy: 67.5% vs. 69.8%, $p>0.05$) 2b. MDA-MB-231 Microscopic Images. There was decreased breast cancer cell density and notable changes in cell morphology at 10 Gy compared to 0 Gy. There was even further reduction in cells at 20 Gy compared to 10 Gy. There was no notable difference in cell density between breast cancer cells pre-treated with WR-1065 compared to the controls at 0 Gy, 10 Gy, and 20 Gy, respectively.

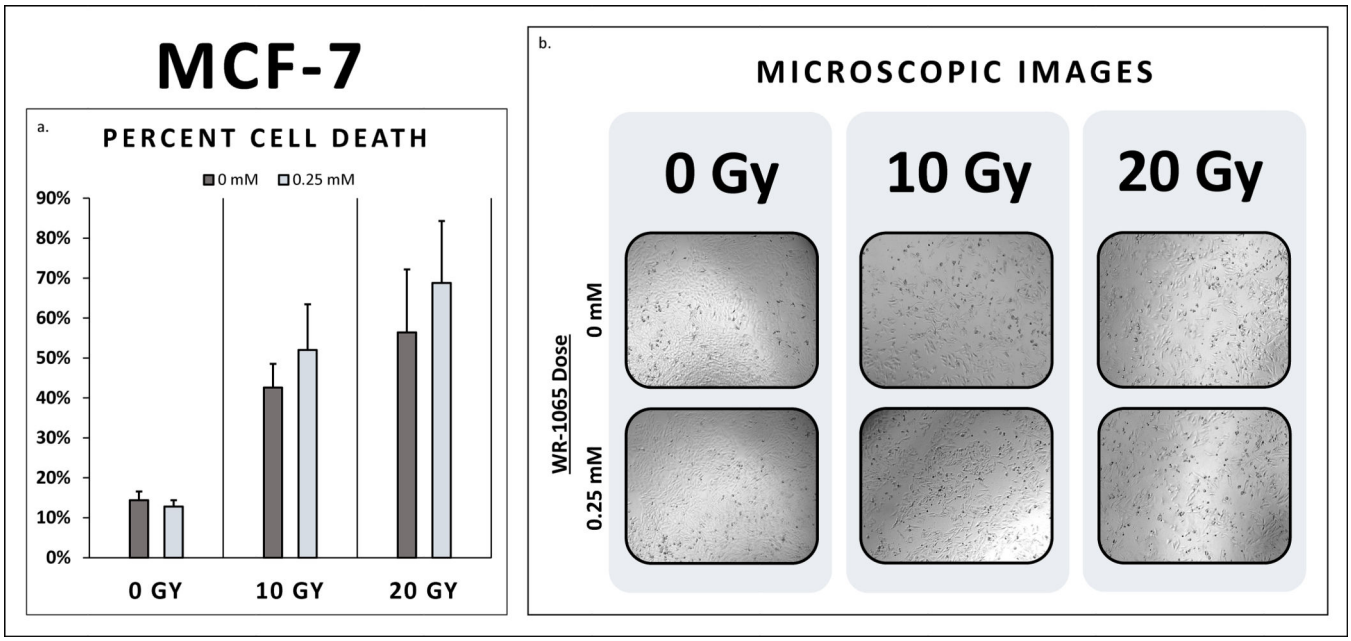

Figure 3:

3a. MCF-7 Percent Cell Death. Cell death did not differ between MCF-7 breast cancer cells pretreated with WR-1065 and their untreated controls at 10 Gy and 20 Gy. Breast cancer cells pretreated with radio-protective doses of WR-1065 attained the same level of cell death as untreated breast cancer cells. (10 Gy: 52.0% vs. 42.6%, $p>0.05$; 20 Gy: 68.8% vs. 56.4%, $p>0.05$) 3b. MCF-7 Microscopic Images. There was decreased breast cancer cell density and notable changes in cell morphology at 10 Gy compared to 0 Gy, with an even further reduction in cells at 20 Gy compared to 10 Gy. There was no notable difference in cell density between breast cancer cells pre-treated with WR-1065 compared to the controls at 0 Gy, 10 Gy, and 20 Gy, respectively.

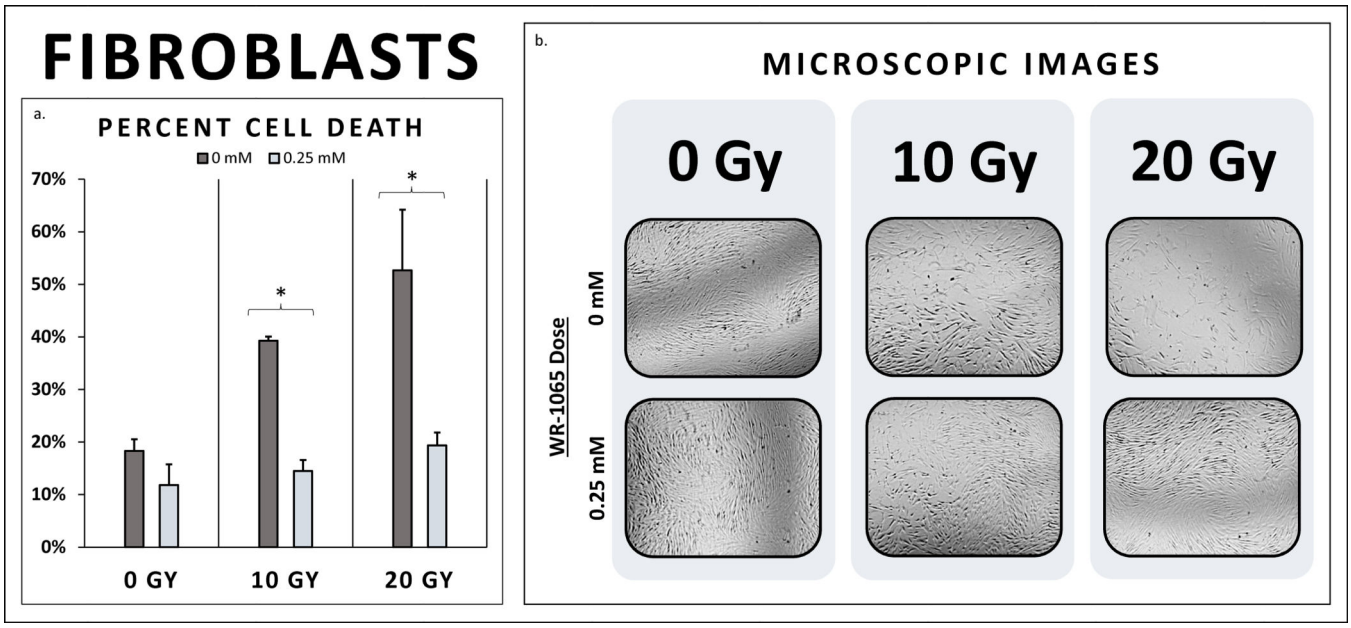

Figure 4:

4a. Fibroblast Cell Death. Healthy fibroblast cells pre-treated with WR-1065 demonstrated a significant reduction in cell death compared to untreated controls at 10 Gy (14.5% vs. 39.3%, $p<0.001$). WR-1065 pre-treatment also preserved healthy FF cell viability compared to untreated controls at 20 Gy (19.4% vs. 52.7%, $p=0.008$). 4b. Fibroblast Microscopic Images. Untreated fibroblasts at 10 Gy show decreased cell density compared to untreated controls at 0 Gy. There is even further reduction in cell density for untreated FF cells at 20 Gy. FF cells pre-treated with WR-1065, however, demonstrate marked preservation of FF cell density. There is marked retention in cells compared to untreated FF at both 10 Gy and 20 Gy. WR-1065 pre-treatment preserves FF cell density, to levels similar to non-irradiated (0 Gy) controls.

Table 1:

Immunohistochemistry Characterization of Breast Cancer Cell Lines under Investigation

Breast Cancer Cell Line	Immunohistochemistry Markers		
	ER+	PR+	Her2+
MDA-MB-231	No	No	No
MDA-MB-468	No	No	No
MCF-7	Yes	Yes	No

Breast cancer cell lines investigated in this study, characterized by immunohistochemistry markers: Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (Her2).

Table 2:

Percent Cell Death According to Treatment Condition

	Cell Death at 0 Gy			Cell Death at 10 Gy			Cell Death at 20 Gy		
	WR-1065 Dose		P-value	WR-1065 Dose		P-value	WR-1065 Dose		P-value
Cell Line	0 mM	0.25 mM		0 mM	0.25 mM		0 mM	0.25 mM	
FF	18.3%	11.8%	0.15	39.3%	14.5%	0.000	52.7%	19.4%	0.01
MDA-MB-468	10.3%	11.4%	0.39	52.8%	54.6%	0.895	60.4%	63.1%	0.66
MDA-MB-231	19.6%	14.3%	0.77	50.9%	61.4%	0.408	69.8%	67.5%	0.89
MCF-7	14.4%	12.8%	0.05	42.6%	52.0%	0.102	56.4%	68.8%	0.55

There was no significant difference in cell death between breast cancer cells (MDA-MB-468, MDA-MB-231, MCF-7) treated with 0.25 mM WR-1065 prior to XRT compared to their untreated controls at 0 Gy, 10 Gy, and 20 Gy. This same dose of WR-1065 demonstrated a significant decrease in FF cell death compared to untreated FF controls at 10 Gy and 20 Gy.