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SIMVASTATIN DOES NOT SENSITIZE IBC3 HER2+ INFLAMMATORY BREAST CANCER BRAIN METASTASES TO WHOLE BRAIN IRRADIATION IN AN IMMUNOCOMPROMISED MOUSE MODEL

Swaminathan Kumar

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A

THESIS

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Swaminathan Kumar, B.Tech.

Houston, Texas

August 2018
Simvastatin Does Not Sensitize IBC3 Her2+ Inflammatory Breast Cancer Brain Metastases to Whole Brain Irradiation in an Immunocompromised Mouse Model

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Retrospective data analysis suggests that inflammatory breast cancer (IBC) patients who take statins have better locoregional control after radiotherapy than those who do not [23]. Our lab has previously demonstrated that simvastatin radiosensitizes IBC cells in vitro [23], and brain metastases have strong expression of cholesterol-regulation genes compared to lung metastases in vivo [unpublished]. Delaying whole-brain irradiation (WBI) beyond 21 days is insufficient to reduce the incidence of brain metastases (developed by injecting IBC3 cells through the tail vein) in our mouse model because even high rates of cell killing leave substantial cell volume in established metastases [unpublished].

With the above data, I hypothesized that the combination of simvastatin and whole-brain radiation will reduce the incidence of established brain metastases in vivo.

I performed two in vivo experiments—in the first, a single-fraction, 10-Gy WBI dose was used, and in the second experiment, a 9-Gy WBI dose was given in 3 fractions of 3 Gy each. The simvastatin dose was kept constant and was mixed with drinking water in both experiments. Brain metastatic lesions were quantified by stereo-microscopy. Brains were cryo-sectioned into mirror sections for mass spectrometry-based tissue imaging and H&E staining. There was a significant reduction (P < 0.05) in brain metastatic burden in the group treated with 10 Gy WBI alone compared to no treatment. Simvastatin
by itself did not significantly reduce the brain metastatic burden compared to no treatment. There was no significant reduction in brain metastatic burden in the combination group compared to the no-treatment or radiation-alone group. There was no significant difference in the incidence of brain metastasis between any of the four treatment groups. Repeating the experiment with a 9 Gy–dose of WBI given in 3 fractions of 3 Gy each also demonstrated no synergy between simvastatin and radiation in this model.

Mass spectrometry-based tissue imaging revealed that treatment with 10 Gy radiation (both as a single-agent and in combination with statins) increased cholesterol (based on its m/z value of 369.35) levels compared to the no-treatment or simvastatin-alone group. Mice brain treated with simvastatin alone had low cholesterol levels compared to the no-treatment group, confirming the role of statins in inhibiting cholesterol biosynthesis. Contrary to my hypothesis, the metastatic burden did not correlate with cholesterol levels in the brain parenchyma for any of the treatment groups.

Thus, simvastatin failed to radiosensitize brain metastases in our model, consistent with our published mathematical model [35] and a recently published clinical trial [28].
TABLE OF CONTENTS

Approval Sheet .................................................................................................................. 1

Title Page ......................................................................................................................... 2

Abstract ............................................................................................................................. 3

Table of Contents ............................................................................................................ 5

List of Figures .................................................................................................................. 7

List of Tables ................................................................................................................... 10

Chapter 1: Introduction ................................................................................................... 11

1.1 Breast Cancer.............................................................................................................. 11

1.2 Inflammatory Breast Cancer...................................................................................... 11

1.3 Treatment Options for Breast Cancer.................................................................... 12

1.4 Metastatic disease ..................................................................................................... 14

1.5 Brain Metastasis ....................................................................................................... 16

1.6 Breast Cancer Brain Metastasis ............................................................................. 17

1.7 IBC Brain Metastasis mouse model ....................................................................... 18

1.8 Treatment Strategies for Breast Cancer Brain metastasis ................................... 18
Chapter 2: Simvastatin Does Not Sensitize Her2+ IBC3 Inflammatory Breast Cancer Brain Metastases to Whole Brain Irradiation in an Immunocompromised Mouse Model ................................. 23

2.1 Background .................................................................................................................. 23

2.2 Hypothesis & Specific Aims ..................................................................................... 25

2.3 Materials and Methods ............................................................................................. 26

2.4 Results ....................................................................................................................... 37

2.5 Discussions and Future Directions .......................................................................... 58

Chapter 3: Mass spectrometry based tissue imaging technology to understand the cholesterol distribution and its perturbations by statin in brain metastatic lesions and the brain tumor microenvironment ................................................. 62

3.1 Background ................................................................................................................ 62

3.2 Materials and Methods ............................................................................................. 63

3.3 Results ....................................................................................................................... 67

3.4 Discussions & Future Directions .............................................................................. 73

Chapter 4: Overall Conclusions ...................................................................................... 77

Bibliography .................................................................................................................. 80

Vita ................................................................................................................................... 90
LIST OF FIGURES

Figure 1: Mouse Whole Brain Irradiation setup .................................................................33

Figure 2: Statin-plus-10 Gy single-dose WBI experimental design ................................................35

Figure 3: Statin-plus-9 Gy WBI dose given in 3 fractions experimental design .................................................................36

Figure 4: Representative H&E images of no-treatment and 10-Gy WBI mice with brain metastases…………………………………………………………………………………………………………………37

Figure 5: Representative H&E image of mice brain with radiation induced necrosis......38

Figure 6: Representative H&E images of 1-year-old SCID-beige mice irradiated with different WBI doses ……………………………………………………………………………………………………………………………39

Figure 7: Representative H&E images of 9-week-old SCID-beige mice (without cancer-cell injection) irradiated with different WBI doses ..........................................................41

Figure 8: Representative H&E images of IBC3 brain-metastasis SCID-Beige mouse models irradiated with different WBI dose …………………………………………………………………………………………………………………………………42

Figure 9: Tumor burden for the no-treatment, simvastatin-alone, 10 Gy WBI-alone, and simvastatin–plus–10 Gy WBI treatment groups..........................................................44

Figure 10: Brain metastasis incidence for no-treatment, simvastatin-alone, 10 Gy WBI–alone and simvastatin–plus–10 Gy WBI treatment groups ………………………………………………………………………………………………………………………………………46
Figure 11: Number of brain metastatic lesions for no-treatment, simvastatin-alone, 10 Gy WBI–alone and simvastatin–plus–10 Gy WBI treatment groups ........................................................................................................................................................................47

Figure 12: Representative fluorescence–bright field overlay images of no-treatment, simvastatin-alone, 10 Gy WBI–alone and simvastatin–plus–10 Gy WBI treatment groups. ........................................................................................................................................................................................................................................48

Figure 13: WBI dose-dependent reduction of IBC3 Brain metastatic burden .........................50

Figure 14: Hypothesis for hyper-fractionated low dose WBI for combinatorial effects with simvastatin ..............................................................................................................................................................................................................51

Figure 15: Tumor burden for no-treatment, simvastatin alone, 9 Gy total WBI dose given in 3 fractions alone and the combination group ........................................................................................................................................................................53

Figure 16: Brain metastasis incidence for no-treatment, simvastatin alone, 9 Gy total WBI dose given in 3 fractions alone, and the combination group ........................................................................................................................................................................55

Figure 17: Number of brain metastatic lesions for groups of no-treatment, simvastatin alone, 9 Gy total WBI dose given in 3 fractions alone, and combination treatment...........................................................................................................................................................................56

Figure 18: Mass Spectrometry Imaging work flow ...........................................................................................................................................................................................................................................66

Figure 19: Cholesterol distribution in the brain parenchyma of no-treatment group (N=4) ..............................................................................................................................................................................................................................68
Figure 20: Cholesterol distribution in the brain parenchyma of statin alone treated group (N=5) ..........................69

Figure 21: Cholesterol distribution in the brain parenchyma of 10 Gy alone treated group (N=3) ..........................70

Figure 22: Cholesterol distribution in the brain parenchyma of 10 Gy-plus-statin treated group (N=6) ..........................71
LIST OF TABLES

Table 1: Simvastatin reduces brain metastasis incidence 10 days after WBI and 31 days IBC cell injection .....................................................................................................................................................38

Table 2: Statistical analysis for tumor burden across no-treatment, simvastatin-alone, 10 Gy WBI–alone, and simvastatin–plus–10 Gy WBI treatment groups............................................44

Table 3: Statistical analysis for number of brain metastatic lesions per mouse across no-treatment, simvastatin-alone, 10 Gy WBI–alone, and simvastatin–plus–10 Gy WBI treatment groups..................................................................................................................47

Table 4: Statistical analysis for tumor burden across groups of no-treatment, simvastatin alone, 9 Gy WBI dose given in 3 fractions alone, and combination treatment............................................................................................................................................52

Table 5: Statistical analysis for the number of brain metastatic lesions per mouse across groups of no-treatment, simvastatin alone, 9 Gy total WBI dose given in 3 fractions alone, and combination treatment ...............................................................................................................................................57
Chapter 1: Introduction

1.1 Breast Cancer

Breast cancer is the most prevalent cancer type among American women and is the second most common cause of cancer-related death in this population. In 2018, it is estimated that there will be 266,120 newly diagnosed cases of breast cancer, and 40,920 breast cancer-related deaths (1). There are different classifications of breast cancer. Clinically, breast cancer is commonly categorized based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (2). Tumors are typically classified as hormone receptor positive (defined as positive for ER and or PR), HER2+ (regardless of ER and PR expression), or triple-negative (TN), which express neither ER, PR, nor HER2 (37). Molecular classifications of breast cancer based on intrinsic gene expression signatures have also been developed, but these are not in routine clinical use (2, 45). HER2+ and TN breast cancers are associated with higher risks of local and regional recurrence and distant metastasis compared to hormone receptor positive breast cancers.

1.2 Inflammatory Breast Cancer

Inflammatory breast cancer (IBC) is a clinically-diagnosed subtype of breast cancer that accounts for 1-4% of all breast cancers. While non-inflammatory breast cancers often present with a palpable mass or are detected by screening mammography, IBC typically appears as a red and swollen breast with changes in the overlying skin (commonly called “peau d’orange”), with or without a palpable mass (3). IBC is the most lethal form of
breast cancer; indeed, approximately 30% of patients with IBC have metastatic disease at the time of diagnosis. IBC is commonly HER2+ or TN, though hormone receptor positive IBC is not rare.

1.3 Treatment Options for Breast Cancer

The approach to treating breast cancer usually involves surgery, radiation therapy and systemic therapies. Surgical options include segmental mastectomy ("lumpectomy") in which the tumor and a small margin of normal breast tissue are removed, or mastectomy, which entails completely removing the breast. Radiation therapy is typically administered as external beam radiation therapy to the whole breast or chest wall. Systemic therapy may include chemotherapy, hormonal and/or targeted therapy, depending on stage and tumor subtype. The sequencing of surgery, radiation therapy, and systemic therapy largely depends on the clinical stage at diagnosis. For patients with early-stage breast cancer, surgery is typically performed first so that the cancer can be pathologically staged. For early-stage patients who opt for lumpectomy, adjuvant (after surgery) radiation therapy is indicated. Radiation therapy is rarely required for early-stage patients who elect mastectomy. For patients with more advanced disease at diagnosis, including those who are found to have involved lymph nodes, neoadjuvant (before surgery) systemic therapy is usually given to reduce disease burden and hopefully eliminate micrometastatic disease that may already exist. Following systemic therapy, patients then go to surgery followed by adjuvant radiation therapy.
Systemic therapies may affect the entire body and can be given either orally or intravenously to a patient. Neoadjuvant systemic therapy is used to shrink tumors so that surgery is a more feasible option, and may allow patients who would have required mastectomy to undergo lumpectomy instead. Neoadjuvant systemic therapy also allows oncologists to determine the response of the primary tumor to treatment. Patients who have a favorable response to neoadjuvant systemic therapy are known to have an improved prognosis compared to patients who have a poor response or progress on treatment (46, 47). In patients who receive surgery upfront, adjuvant systemic therapy can be used to eliminate residual cancer cells that remain at the primary site or may have migrated to a distant part of the body.

Chemotherapy describes a group of cytotoxic systemic therapies that are used to treat breast cancer. The underlying mechanism behind most chemotherapy agents is to kill cancer cells that are rapidly dividing; however, this approach can also be lethal to normal cells which undergo rapid cell division. These drugs can be classified as one of the following: alkylating agents, anti-metabolites, anti-microtubule agents, topoisomerase inhibitors, and anthracyclines. The most common chemotherapeutic backbone in breast cancer treatment involves use of doxorubicin (an anthracycline), cyclophosphamide (an alkylating agent), and paclitaxel (a microtubule poison).

Targeted therapies are routinely used for the treatment of HER2+ and hormone receptor positive breast cancers. Agents like the anti-HER2 monoclonal antibodies trastuzumab and pertuzumab, and the HER2 small molecule antagonist lapatinib have revolutionized the treatment of both early-stage and advanced HER2+ breast cancer and
have markedly improved outcomes for these patients. Similarly, therapies targeting estrogen production and/or the estrogen receptor (e.g., selective estrogen receptor modulators (SERMs) like tamoxifen, aromatase inhibitors like exemestane, and gonadotropin releasing hormone agonists like goserelin or leuprolide) are useful in the treatment of hormone receptor positive disease. Although multimodal therapies have improved treatment outcomes and cure rates for breast cancer patients with early-stage disease, the development of metastatic disease remains the major driver of breast cancer related mortality. Novel approaches to treat metastatic disease are still needed.

1.4 Metastatic disease

Cancer cells that invade distant organs from the primary tumor site are part of a process called metastasis. It is this process that is responsible for most cancer deaths even though there have been improvements in detection, tumor removal and treatment. Metastatic disease is generally resistant to conventional methods of treatment (4). Clinically, metastasis is a major hurdle to achieving significantly better patient outcomes, thus making new and improved therapies for treating it an important part of cancer research.

Metastases arise from a set of unique cells which reside within the primary tumor (38). The metastatic process is complex, and the tumor cells that form metastases are heterogenous clones with different mutations. Only a small percentage of tumor cells that are circulating in the bloodstream or migrate to regional lymph nodes will actually develop into metastatic lesions (5, 39).
Metastasis is a multi-step process, and there remains some controversy regarding the mechanisms of several steps. In one model, metastasis begins with a cell undergoing a process called the epithelial-mesenchymal transition (EMT) in which it loses adhesion with its neighbors (characterized by loss of E-Cadherin expression), invades through the basement membrane, migrates to a blood or lymphatic vessel, and then enters the systemic circulation (intravasation). EMT is a critical component of this model (48). Another model proposes a process called collective migration, whereby small aggregates of cells collectively invade and migrate together, resulting in polyclonal metastases (49). It is possible that both of these mechanisms contribute to the development of inflammatory breast cancer metastases.

Cancer cells can reach the systemic circulation through the hematogenous route (involving blood vessels) or can enter into the lymphatic vessels (lymphatic circulation) and reach a nearby lymph node and drain into the blood vessels. Once the tumor cells have reached the systemic circulation, they can circulate throughout the body. Cancer cells can enter the tissue of a nearby or distant organ, which is referred to as extravasation. In order to migrate inside the tissue of the secondary site, the cells could move between two endothelial cells or pass through one. Once the tumor cells have established themselves within the tissue of the organ, they can begin to develop their own network of blood vessels.

The formation of metastases depends upon the interactions between tumor cells and the surrounding tissue of the secondary organ. The majority of the micro-metastases that form will not grow into lesions that are detectable in the clinical setting (40). Micro-
metastases can be widely distributed throughout the tissues of the organ, so it is very important to try and eliminate them before they become too large, which is detrimental to the structure and function of the secondary organ (40). Eradication of micrometastases is one of the major rationales for the use of neoadjuvant systemic therapy. For a disease like IBC, where the risk of occult metastatic disease is exceedingly high, early introduction of therapy to eradicate these micrometastases likely modifies the course of the disease. Radiation therapy is also used to eradicate micrometastatic disease. Examples include the use of nodal irradiation to clinically-uninvolved lymph node basins, which has been shown to reduce the risk of distant metastasis in breast cancer (50, 51), and even prophylactic cranial irradiation (PCI) in small cell lung cancer (SCLC), which has been shown to reduce the incidence of brain metastasis and improve survival (52, 53).

1.5 Brain Metastasis

Most malignant brain tumors are the result of metastases to the central nervous system (41). Lung cancer (40-50%), breast cancer (15-25%), and melanoma (5-20%) are the top three sources of brain metastasis (6). While brain metastases can develop at any point during the course of disease, metastatic brain lesions typically appear at a later point in time after the cancer has spread to other organs (6).

During the process of brain metastasis, tumor cells may proliferate within the brain capillaries before extravasating into the brain tissue. Endothelial cells lining the brain vasculature may aid the growth and extravasation of tumor cells (7). The blood-brain barrier (BBB), which acts as a barrier between the peripheral blood and the fluid in the brain, may help the tumor cells enter into the brain parenchyma when it is breached.
It is possible that the cancer cells could exit the blood vessel, cross over into the brain tissue and then proceed to die or become dormant. Once cells are in the brain tissue, they will encounter both microglia and astrocytes, which can be conducive to tumor growth and treatment resistance (42-44).

1.6 Breast Cancer Brain Metastasis

As mentioned previously, brain metastases can be formed from breast tumors. When considering all breast cancer patients, only about 5% will develop brain metastases. However, the risk increases greatly for patients with advanced disease at presentation, and even more so for patients who already have extracranial metastatic disease. Approximately 25-35% of stage IV (metastatic) HER2+ patients and 40-45% of stage IV TN patients have or will develop brain metastases (9, 10). It has been reported that overexpression of the HER2 protein in a breast cancer cell line that is preferential to the brain increased the quantity of large brain lesions significantly, but it did not increase the number of micro-lesions (11). HER2 may play a role in establishing the population of brain metastasis, but it does not affect the initial stages of brain metastasis (8). These data suggest that HER2 is an important driver of increase in brain metastatic burden (larger lesions) and does not increase the incidence or the number of brain metastatic lesions.

Brain metastasis in breast cancer is generally a terminal event, and expected survival time is very low (~1 year) for patients who have developed brain metastases. Strategies to improve outcomes for breast cancer patients with brain metastases are a tremendous unmet need.
1.7 IBC Brain Metastasis mouse model

Animal models are very important for understanding the biology of brain metastases and for creating new therapies aimed at treating or preventing this phenomenon. One group found that a triple-negative breast cancer cell line only reaches the brains of mice approximately 42% of the time (12). To better understand mechanisms of brain metastasis in IBC, our group has developed a model using a HER2+ inflammatory breast cancer cell line known as IBC3 that goes to the brain approximately two-thirds of the time following tail vein injection into immunocompromised SCID-beige mice (13). We have developed GFP-labeled IBC3 cells that allow us to quantify the number and burden of tumor cells in the brain using fluorescence based stereo microscopy. The veracity of this model was confirmed when the genetic knockdown of a brain metastatic specific microRNA (miR-141) resulted in a nearly complete absence of brain metastatic lesions. Since the IBC3 cell line has a high propensity for forming brain metastases using our tail vein injection model, it was used for in vivo experiments discussed in chapter 2. This model could be used to mechanistically study IBC brain metastases and to evaluate new treatment approaches for IBC brain metastasis.

1.8 Treatment Strategies for Breast Cancer Brain Metastasis

To date, there are generally no curative treatment options for breast cancer metastasis to the brain. Palliative treatment options depend on the patient’s burden of extracranial disease, response to prior therapies, the possibility of surgery, the quantity, location, and size of the lesions, as well as the patient’s expected prognosis. Depending
on these factors, patients may undergo surgical resection, whole-brain radiation therapy (WBRT), stereotactic radiosurgery (SRS), or intrathecal chemotherapy or targeted therapy. WBRT involves giving several low doses of radiation to the entire brain, whereas SRS employs high-dose focal irradiation in one or a few fractions. In the U.S., currently there are no FDA (Food & Drug Administration) approved systemic or intrathecal therapies specifically for brain metastasis arising from breast cancer, however, several agents are used off-label and there are ongoing clinical trials (14).

Surgical resection, WBRT, SRS, and intrathecal therapies are palliative options and rarely result in permanent control of disease within the central nervous system (CNS). For patients who have good control of extracranial metastases, progression of CNS metastatic disease is a major driver of morbidity and mortality. There are no solid guidelines for treating patients with recurrent brain lesions. The National Comprehensive Cancer Network (NCCN) recommends that patients with stable systemic disease receive surgery, re-irradiation or chemotherapy for their brain metastasis. If a patient’s systemic disease has progressed, then treatment options might include best supportive care or re-irradiation (14). As is the case for treatment-naïve brain metastases, strategies to control recurrent or progressive brain metastases are needed.

Chemotherapy options for patients with brain metastases are somewhat limited for several reasons. First of all, patients usually undergo several rounds of chemotherapy before they present with brain metastasis which could be chemoresistant. Secondly, clinical trials may not include patients with brain metastases (15). CNS penetration of many systemic agents is poor, due to the presence of the blood brain barrier (BBB),
composed of tight junctions connecting the endothelial cells. There are many active transporters inside the cells which pump all but small lipophilic substances back into the peripheral circulation (16). When a lesion is approximately 1-2 millimeters in diameter, the BBB is compromised and becomes leaky (17). Even though the BBB is compromised and chemotherapeutic agents enter the brain, there is usually not enough drug near the lesion to make a difference in most cases.

Trastuzumab is a systemic agent used to treat HER2+ breast cancer. Trastuzumab dramatically reduces the incidence and progression of extracranial metastatic disease, but since it does not effectively cross the BBB, the CNS is a sanctuary site for HER2+ breast cancer metastases. Accordingly, HER2+ brain metastases may become apparent years after the primary cancer is cured. Strategies to eradicate micro-metastatic breast cancer cells in the CNS that are anatomically shielded from trastuzumab and other systemic therapies may improve outcomes for breast cancer patients. The use of prophylactic cranial irradiation (PCI) in SCLC is an example of this approach (18).

PCI is a preventative strategy that employs whole-brain irradiation in patients who are at risk for developing brain metastases. There is an elevated risk for brain metastases in some advanced primary tumors such as breast cancer, acute lymphoblastic leukemia and small-cell lung cancer (54). Even though new therapies are being utilized, patients with brain metastases may not survive more than a year after the brain metastases have been detected. Undetected brain micro-metastases can be problematic for cancer patients. These undetected brain lesions could be an opportunity for employing treatments that could alter the environment of the brain so that there can be a reduction
or hindrance to brain metastasis formation. This prophylactic approach may even help to reduce the cognitive problems that patients’ sometimes experience with conventional brain metastasis treatments. While WBRT is commonly used in the treatment of patients with clinically evident brain metastases from any solid tumor, or in leukemia patients with CNS involvement, to date, prophylactic irradiation of the whole brain is only formally recommended for patients diagnosed with limited-stage small cell lung cancer (LS-SCLC) (NCCN guidelines).

Currently, there are no large-scale studies to help evaluate the effectiveness of prophylactic cranial irradiation in breast cancer patients. Since PCI has not been used as a standard of care for breast cancer patients who are at high risk for brain metastasis, inevitably, many of these individuals develop brain metastasis during the course of their disease. In order to develop a treatment that effectively reduces the incidence of brain metastasis in IBC with a favorable toxicity profile, we must be able to correctly identify the patients who are high risk of developing brain metastasis, understand the temporal patterns of brain metastasis seeding, and optimize the radiation dose to effectively eradicate tumor cells but maximize preservation of the normal brain.

One strategy to improve the therapeutic index for whole brain irradiation is to utilize a radiosensitizer that will preferentially increase the efficacy of radiation therapy in tumor cells while not amplifying the adverse effects of radiation therapy in normal brain tissue. Based on previous studies in our laboratory linking cholesterol metabolism with radiosensitivity in IBC, my thesis project looked at whether we can use hydroxymethylglutaryl-CoA (HMC-CoA) reductase inhibitors (“statins”), which inhibit
cholesterol biosynthesis, for treating late-stage inflammatory breast cancer brain metastasis using our IBC3 model system.
2.1 Background:

Women in western countries have a greater chance of developing breast cancer than women in other countries. Diets in the United States and other western countries are typically high in cholesterol and fat, and studies have shown that there is a link between obesity and breast cancer. It has also been shown that one of the risk factors for breast cancer is high cholesterol levels in the blood (19). Statins are drugs which reduce blood cholesterol levels by targeting a key enzyme in the cholesterol biosynthesis pathway, HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase. Specifically, statins reduce mevalonate levels in cells by inhibiting the HMG-CoA reductase involved in the conversion of HMG-CoA to mevalonate. Mevalonate is an intermediate molecule converted into cholesterol by multiple enzymes in the cholesterol biosynthesis pathway. The effects of statin on breast cancer progression and on breast cancer patients diagnosed with high cholesterol levels have been studied. Statins have been shown to reduce breast cancer occurrence and improve outcomes.

2.1.1 Studies from other groups which helped us to form our hypothesis:

In a ten-year study conducted by researchers in Denmark, patients who used simvastatin had far fewer recurrences than patients who did not use the drug (20). Another study looked at cholesterol metabolism differences between non-inflammatory and inflammatory breast cancer cells. The study found that both non-IBC and IBC cells have a higher cholesterol content compared to normal mammary epithelial cells. In
cholesterol-depleted microenvironments in vitro, IBC cells could also produce their own cholesterol whereas non-IBC cells could not (21). Studies from other groups have shown that glioblastoma (GBM) cells are dependent on astrocytes in the brain for cholesterol and targeting this metabolic dependency of GBM cells can have therapeutic effects (22).

2.1.2 Studies from our group which helped me to form my hypothesis:

From a retrospective patient data analysis, it was found that patients taking statins had better primary tumor control after radiotherapy compared to those who did not (23). Our lab has previously demonstrated that statins influence inflammatory breast cancer (IBC) cells and the tumor microenvironment. First, we found that simvastatin radiosensitizes IBC cells in vitro to gamma radiation, likely by targeting stem-like and progenitor cells (23). Second, our lab has shown that statins and lipoproteins impact the supporting stromal cells, namely mesenchymal stem cells and macrophages, further influencing radioresistance in IBC (24). Third, our lab has found using gene expression microarrays that IBC brain metastases have strong expression of cholesterol regulation genes compared to lung metastases using [unpublished]. Fourth, our lab has shown that statins are able to inhibit IBC metastasis formation by upregulating a tumor suppressor, FOXO3a (25). Fifth, our lab has found that delaying whole-brain radiotherapy (4 Gy) beyond 21 days is insufficient by itself to reduce the incidence of IBC brain metastases using a tail-vein injection mouse model (discussed in Section 1.7), because even high rates of cell killing leave substantial cell volume in established metastases [unpublished]. Since WBI by itself was not sufficient to reduce the incidence of established brain metastasis,
my project sought to combine a radiosensitizer with WBI to treat established brain metastases.

2.2 Hypothesis & Specific Aims:

Based on ours and other groups’ findings, we hypothesized that the combination of simvastatin and whole-brain radiation therapy (WBRT) would eradicate established brain metastases in vivo, implying a synergy between the intrinsic cancer-cell killing and suppression of pro-tumor brain parenchyma.

To test the above hypothesis, I had three specific aims:

Specific Aim 1: To optimize the whole brain radiation dose in SCID-beige mice brain metastasis model

Specific Aim 2: To test the combinatorial effects of simvastatin and optimized whole-brain radiation dose in an IBC brain metastasis mouse model

Specific Aim 3: To adapt mass spectrometry–based tissue imaging (MSI) methodology to understand the cholesterol distribution and its perturbations by simvastatin in brain metastatic lesions and the brain tumor microenvironment

Specific Aim 1 and 2 is discussed in this chapter. Specific Aim 3 will be discussed in Chapter 3.
2.3 Materials and Methods:

(*- Swaminathan Kumar, **- Richard Larson, ***- Shane Stecklein)

A. Cell line and culture conditions

For the in vivo studies we used GFP-labeled MDA-IBC3, a HER2+ inflammatory breast cancer patient-derived cell line generated in our lab and used previously (13). MDA-IBC3 cells were grown in Ham’s –F12 media with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic cocktail, 5 μg/mL insulin and 1 μg/mL hydrocortisone. Cells were maintained at 37°C with 5% CO₂ and 95% O₂ (S.K.). Cells were checked for mycoplasma contamination using Lonza’s MycoAlert™ Mycoplasma Detection Kit before using them for any experiments (S.K.). Whenever contaminated with mycoplasma, they were treated with plasmocin (S.K.). Only mycoplasma-free cells were used for in vitro and in vivo experiments (S.K.). Media was changed once every three days for IBC3 cells (S.K.). IBC3 cells have a doubling time of 76 hours. When they were 70% confluent, they were passaged at a 1:4 ratio once every week before injection into the mice (S.K.).

B. Mouse strain used:

Three-week-old SCID/Beige mice were purchased from Harlan, USA and housed according to institutional guidelines of the University of Texas MD Anderson Cancer Center under the Institutional Animal Care and Use Committee approved protocol (00001063-RN00). Mice were monitored regularly and were weighed weekly after tumor-cell injection (S.K., R.L.).
C. Intravenous Tail Vein Injection

IBC3 cells were grown to 60-70% confluency and were trypsinized using 0.25% trypsin-EDTA and neutralized with Fetal Bovine Serum (FBS) present in the culture media (S.K.). Cells were washed twice with PBS before counting and were counted twice using two cell counters, Nexcelom Bioscience’s Cellometer™ Auto T4 Bright Field Cell Counter and Bio-Rad’s TC20™ Automated Cell Counter (S.K., R.L.). The average of cell counts from the two cell counters were taken as the number of cells present in 1 mL of the cell suspension (stock concentration) (S.K.). Stock concentration was adjusted to 2.5 x 10⁶ cells/mL using PBS and added to 1.5 mL sterile Eppendorf tubes after mixing the cell suspension (S.K.). Cells were placed on ice before injection into SCID/Beige mice. 200 μL of cell suspension (500000 cells) were injected through the tail vein of 6-week-old mice using a 30-gauge needle. Cell injections were done by a veterinary medicine technologist at the North Campus Animal Facility of MD Anderson Cancer Center.

We chose 500000 IBC3 cells because we knew from previous experiments in our lab that 500000 cells form brain metastases in 9 weeks in the maximum number of mice (70 – 100%) (13). One previous lab member had done serial dilutions of IBC3 cells (500000, 250000, 50000, 5000, and 500) and injected 10 mice in the tail vein, each with a particular number of cells. In a 9-week period without any therapeutic intervention, the groups injected with 500000, 250000, 50000, 5000, and 500 cells formed brain metastases at incidence rates of 10/10, 9/10, 3/10, 0/10, and 0/10 respectively. Based on this data, we decided to inject 500,000 cells for my experiment so that the maximum number of mice
could form brain metastases by end of the 9-week period, when all the mice, irrespective of treatment group, were sacrificed.

**D. Statin and Whole Brain Irradiation (WBI) treatment plan:**

Four weeks after cell injection, mice were irradiated with 4 Gy, 6 Gy, 8 Gy, 10 Gy or 9 Gy in 3 fractions according to the experimental plan discussed in the results section (S.K., R.L.). From our lab’s unpublished data, we knew that delaying WBI beyond 21 days after cancer cell injection was not sufficient to reduce the incidence of brain metastasis [unpublished data]. To treat established brain metastases with a radiosensitizer (simvastatin) and WBI, we set our treatment time 4 weeks after cell injection (treatment setting). From the initial dose-finding experiments, 10 Gy was chosen as the highest tolerated dose and used for the first simvastatin-plus-WBI experiment (S.K.). The highest tolerated dose was defined as the highest dose at which there was no radiation-induced brain necrosis, which was confirmed by HE staining and pathologist examination. We chose the highest tolerated dose for our experiments as this was more logistically feasible and cost-effective for the proof of concept of combining simvastatin plus WBI. For our second combination study, simvastatin was used with hyper-fractionated (9 Gy in 3 fractions) WBI dose delivered on days 29, 30, and 31 (3 Gy on each day) after cancer cell injection (S.K., R.L.).

For WBI, mice were placed in an anesthesia inhalation chamber containing isoflurane (S.K., R.L.). Anesthetized mice were transferred to the stage in the X-RAD 225 Cx small-animal irradiator ((Figure 1), PRECISION X-RAY, North Branford, CT, USA) (S.K., R.L.). The stage is used to image, plan treatment, and treat each mouse separately. The
stage contains a nose cone through which the mice receive constant supply of isoflurane to keep them anesthetized. After the mouse was placed on the stage, a scout cone-beam computer tomography (CT) image was taken for each of them with a 2.0-mm aluminum filter at 40 kVp and 2.50 mA to manually set the isocenter (S.K.).

The treatment plans were developed by MD Anderson North Campus small-animal imaging core technologists with PilotXRAD 1.10.4 software and was used for irradiating all mice. Different treatment plans were used for single WBI dose of 4 Gy, 6 Gy, 8 Gy, 10 Gy or 9 Gy in 3 fractions (S.K.). Mice were treated with an isocentric technique with equally weighted, opposed lateral 225 keV photon beams using circular fields (shaped to 15 mm diameter with a copper collimator) using a 0.3 mm copper filter to remove ultra-low-energy photons (S.K.). For example, for a single fraction of 10 Gy irradiation, mice received 5 Gy WBI each from two opposing fields that are 180 degrees from each other. A cone beam CT was taken for each animal prior to treatment to facilitate target localization (S.K.). Fields were positioned to minimize irradiating the oral cavity and pharynx. The nominal treatment dose (normalized to 100%) was prescribed to the midplane, assuming a depth of 6 mm from the surface of the animal to the isocenter. X-rays were delivered at 225 kVp and 13.0 mA with the following exposure time from each field of radiation (S.K.)

For 10 Gy – from each field 5 Gy was delivered for 103.88 s
For 8 Gy – from each field 4 Gy was delivered for 83.11 s
For 6 Gy – from each field 3 Gy was delivered for 62.33 s
For 3 Gy – from each field 1.5 Gy was delivered for 31.16 s
For the first simvastatin-plus-WBI experiment (Figure 2), a total of 70 mice was used. The control consisted of 15 mice treated with neither statin nor with WBI and given only plain water. Based on the previous dose of simvastatin that was used in mouse studies from our lab [25], 15 mice received simvastatin alone in their drinking water (15 mg/kg/day). 15 mg/kg/day dose is approximately 25 times more than the amount of simvastatin (40 mg tablet on average) taken orally by a dyslipidemia patient weighing 70 kg. Simvastatin-treated mice began receiving water supplemented with simvastatin at a concentration of 0.04 mg/mL after four weeks of cancer-cell injection. The simvastatin-supplemented drinking water was changed weekly until all mice were euthanized (S.K., R.L.). 20 mice received 10 Gy WBI alone and 20 mice received 15 mg/kg/day statin plus 10 Gy WBI (S.K., R.L.). Mice were euthanized 5 weeks after radiation treatment (S.K., R.L.).

Based on previous experiments carried out in our lab [13], we knew that maximum number of mice in the control group (70 – 100%) will form brain metastasis after 9 weeks of injecting 500,000 IBC cells through the tail vein. We therefore delivered WBI after 4 weeks of cancer injection (treatment setting) and sacrificed all the mice after 5 weeks of radiation (total 9 weeks). We did not perform survival analysis, in which we sacrifice each mouse as it becomes moribund, because our primary goal was to determine if adding simvastatin to WBI reduces the incidence of brain metastasis at a particular time point (5 weeks after cancer cell injection) rather than if simvastatin increased the survival of mice with brain metastases. Brain metastatic status was determined by fluorescent microscopy (S.K., S.S.).
For the second *in vivo* simvastatin plus WBI experiment, the following changes were made from the above experiment plan (Figure 3):

1) We hypothesized that the bioavailability of simvastatin in the brain at the time of irradiation is important for their synergistic effects. We therefore hypothesized arbitrarily that starting simvastatin treatment one week prior (from day 22 after cancer cell injection) to the radiation treatment (day 29, 30, 31) would create synergistic effects between simvastatin and WBI (S.K., W.W.).

2) Fractionated 3 Gy radiation for three consecutive days (day 29, 30, 31) was given to test the hypothesis that simvastatin could affect the sub-lethal repair machinery of the cell and have synergistic effects with radiation-induced damage (S.K., S.S.).

**E. Fluorescent Based Stereo Microscopy**

Five weeks after WBI, all mice were sacrificed and brains were collected in cold PBS (S.K., R.L.). Brain were imaged using a fluorescent-based stereo microscope (Nikon AZ100, Tokyo, Japan) at 20X magnification and 35% of maximum excitation laser intensity (as 100% produced too much background/noise) for identifying metastatic lesions based on the GFP intensity level of the IBC3 cells (S.K., S.S.). Bright-field images without fluorescence were taken to capture the “structure” of each mice brain (S.K., S.S.).

Three researchers, who were not involved in imaging the mice brains, counted the number of brain metastatic lesions per mouse from the stereo microscope GFP images. They were blinded to the treatment groups. For the incidence of brain metastatic lesions (binary variable—presence or absence), the saved images of the top and bottom
of the brain were used and the three researchers’ counts were plotted individually. For the number of brain metastatic lesions, the images of the top and bottom the brain were used and arithmetic mean of three researchers’ counting were plotted for each treatment group. If two metastatic lesions coalesced without clear separation, they were counted as one.

For tumor burden, the threshold between actual GFP signal and background was set initially using ImageJ software (S.K., S.S.). The threshold was set using a mouse brain image from the control group and visually adjusting the background such that only brain metastatic lesions were visible with minimum background signal (S.K., S.S.). The same threshold intensity value was applied for all the images automatically using the ImageJ macro program (S.K., S.S.). Tumor burden was calculated by dividing the integrated (top and bottom) GFP pixel area by the integrated total brain area (S.K.).

**F. Statistical Analysis**

Statistical calculations were performed in GraphPad Prism 7 and SPSS v24. All tests were two-sided, and p-values less than 0.05 were considered statistically significant (S.K., S.S.).

**G. Tumor burden and Number of Brain Metastatic lesions data analysis**

Inter-rater reliability was calculated using the two-way random intraclass correlation coefficient (ICC) for average measures (S.S.). The number of metastatic lesions and tumor burden were compared pairwise using the non-parametric Dunn’s multiple comparisons test (S.K.).
H. Brain metastasis Incidence data analysis

Inter-rater reliability was calculated using Fleiss’ Kappa coefficient for binary categorical measures (S.S.). Binary incidence of brain metastasis was compared using the Fisher-Freeman-Halton test separately for each rater’s observation (S.S.).

Figure 1: Mouse whole-brain irradiation setup. A) X-RAD 225Cx, an irradiator dedicated to small-animal irradiation was used for mouse whole-brain irradiation. B) Mice
were kept anesthetized using isoflurane through the nose cone outlet present in the machine. C) A scout cone-beam CT image was used to set the isocenter, and a copper collimator 15-mm in diameter (Bottom panel of B)) was used to define the treatment field, shown as a red circle in the software. The above figure was adopted from the thesis work of Dr. Dan Smith, Ph.D. student from the Woodward lab.

**Recommended Citation**


http://digitalcommons.library.tmc.edu/utgsbs_dissertations/613
Figure 2: Statin-plus-10 Gy single-dose WBI experimental design. 500,000 GFP-labelled MDA-IBC3 cells per mouse were injected intravenously through the tail vein of 70 SCID-Beige mice. Four weeks after cell injection, a 10 Gy single WBI dose was administered to 40 mice (2 groups.) Statin treatment was started immediately after radiation treatment for 35 mice (2 groups) and continued for 5 weeks. At the end of 5 weeks after radiation treatment, mice from all four groups were sacrificed and their brains and lungs were collected to identify metastatic lesions.
Figure 3: Statin-plus-9 Gy WBI dose given in 3 fractions experimental design.

500,000 GFP-labelled MDA-IBC3 cells per mouse were injected intravenously through the tail vein of 74 SCID-Beige mice. Four weeks (on Day 29, 30, 31) after cell injection, a 9 Gy WBI dose in 3 fractions was administered to 38 mice (2 groups). Statin treatment was started for 37 mice (2 groups) one week before the first radiation dose (from Day 22) and was continued for 5 weeks after irradiation. At the end of 5 weeks after radiation treatment, the mice from all four groups were sacrificed and their brains were collected to identify metastatic lesions.
2.4 Results:

A. Optimization of whole brain radiation dose in SCID-beige mice brain metastasis model

A lab member had previously performed an *in vivo* experiment combining statin plus 10 Gy whole brain radiation treatment (WBRT) in a SCID-beige brain metastasis mouse model. The group treated with simvastatin plus radiation had lower incidence of brain metastases compared to the other treatment groups (no treatment, simvastatin alone, and 10 Gy alone) (Table 1.) While the combination therapy worked as expected, supporting our hypothesis, mice had to be sacrificed much earlier (10 days after radiation treatment) because they were sick. The original planned time point for sacrificing mice was 5 weeks after radiation treatment. Of note, when we examined the HE stains of the no-treatment and radiation-alone groups, there was no brain toxicity (Figure 4, 5) in any of the mice. One possible reason for these outcomes is mucositis of the tongue caused by misdirected delivery of WBI. Since tongues were not collected from this experiment, that possibility could not be ruled out.

![No Treatment Control Brain with Metastasis](image1)

![10 Gy WBI treated Brain](image2)

**Figure 4**: Representative H&E images of no-treatment and 10-Gy WBI mice with brain metastases. Mice with no treatment or 10 Gy WBI showed normal brain
architecture and did not have any necrosis or lymphatic damage. Images were taken at 20X magnification (50% magnified using Leica’s Aperio ImageScope software).

<table>
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<tr>
<td>%</td>
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Table 1: Simvastatin reduces brain metastasis incidence 10 days after WBI and 31 days IBC cell injection. The group treated with simvastatin plus radiation had a lower incidence of brain metastases compared to the other treatment groups (no treatment, simvastatin alone and 10 Gy alone) (p = 0.002, Cochran trend test). Courtesy of Dr. Jay Reddy, previous member of the Woodward lab.

Figure 5: Representative H&E image of mice brain with radiation induced necrosis. The image shows a necrotic area (no cells) caused by radiation. The necrotic region is typically covered by a region which undergoes gliosis to cover the damaged
region. Gliosis process involves the proliferation or hypertrophy of different types of glial cells, namely astrocytes, microglia and oligodendrocytes.

There was no other study which had previously published a tolerated whole-brain radiation dose in SCID-beige mouse model. I optimized the whole-brain radiation dose in SCID-beige mice using the following experiments:

**Experiment 1**: 1-year-old SCID-beige mice were irradiated with an 8-Gy single dose (3 mice per group), a 6-Gy single dose (2 mice per group) and a thrice-fractionated dose of 3 Gy (3 mice per group). No cancer cells were injected into these mice. We used 1-year-old mice for logistical reasons, i.e. to help me learn how to administer WBI using unused old mice available in our animal facility. We did not use 1-year-old mice to study age-related brain toxicities. We wanted to identify the optimal radiation dose with least toxicity to the normal brain. 5 weeks after irradiation, mice were sacrificed and brains were dissected. H&E staining of all irradiated brains revealed that they had normal brain architecture and did not have any necrosis or lymphatic damage (Figure 6). H&E staining results were confirmed with an expert pathologist at MD Anderson Cancer Center.

![Representative H&E images of 1-year-old SCID-beige mice irradiated with different doses](image)

**Figure 6**: Representative H&E images of 1-year-old SCID-beige mice irradiated with different doses.
different WBI doses. Mice irradiated with single dose of 8 Gy, 6 Gy and 9 Gy in 3 fractions showed normal brain architecture and did not have any necrosis or lymphatic damage. Images were taken at 20X magnification (50% magnified using Leica’s Aperio ImageScope software)

Experiment 2. To maintain the same experimental condition as our major in vivo experiments (using 70 – 74 mice) we bought 10 SCID-beige mice, and when they were 9 weeks old, whole-brain radiation was given to them as follows: a 10-Gy single dose (3 mice per group), an 8-Gy single dose (3 mice per group), 6 Gy single dose (2 mice per group), and a 9 Gy WBI dose in 3 fractions (3 mice per group). IBC3 cancer cells were not injected into any of the above 10 mice. Our major in vivo experiments used 9-week-old mice since that is standard procedure in our lab and not because of any age-related effects [13 and unpublished work from Dr. Dan Smith]. We sacrificed all 10 mice 5 weeks after irradiation and collected their brains. H&E staining of all irradiated brains revealed that they had normal brain architecture and did not have any necrosis or lymphatic damage (Figure 7.) H&E staining results were confirmed with an expert pathologist at MD Anderson Cancer Center. Some of the mice had dermatitis after radiation. However, these mice recovered from dermatitis upon Baytril antibiotic treatment. This experiment’s results were surprising since none of the 10-Gy, whole-brain irradiated mice died, unlike in the experiment conducted by a previous lab member (as mentioned above), and all 3 mice survived 5 weeks after radiation.
Figure 7: Representative H&E images of 9-week-old SCID-beige mice (without cancer-cell injection) irradiated with different WBI doses. Normal mice irradiated with single dose of 10 Gy, 8 Gy, 6 Gy, and 9 Gy in 3 fractions showed normal brain architecture and did not have any necrosis or lymphatic damage. Images were taken at 20X magnification (50% magnified using Leica’s Aperio ImageScope software.)

Experiment 3: I wanted to test if creating brain metastases in SCID-beige mice would have a different toxicity profile since experiments 1 and 2 were performed without cancer-cell injection. We injected 9 SCID-beige mice, 6 weeks old, with 500,000 GFP-labelled IBC3-GFP-luc cells per mouse through the tail vein. 21 days after cancer cell injection (for treatment setting), we irradiated 3 mice with 10 Gy alone, 3 mice with 8 Gy, and 3 mice with 6 Gy. Seven out of nine mice, irrespective of the radiation dose given, developed dermatitis and received Baytril treatment for the same. We had 2 control mice which were not injected with cancer cells but received 10 Gy radiation alone. Interestingly, again all the mice survived 5 weeks after radiation treatment, and we sacrificed all 11 mice to collect their brains and check for toxicity. H&E staining of all 11 brains revealed that they had normal brain architecture and did not have any
necrosis or lymphatic damage (Figure 8.) H&E staining results were confirmed with an expert pathologist at MD Anderson Cancer Center. We therefore chose 10 Gy as the maximum tolerated dose among all the doses used in the above experiments for our major in vivo studies combining simvastatin with whole-brain irradiation in IBC3 brain metastasis SCID-Beige mouse models. We chose the highest tolerated dose as this was more logistically feasible and cost-effective for the proof of concept of combining simvastatin plus WBI.

Figure 8: Representative H&E images of IBC3 brain-metastasis SCID-Beige mouse models irradiated with different WBI dose. 6-week-old SCID-beige mice were injected with 500,000 GFP-labelled IBC3 cells per mouse through the tail vein. 21 days after cancer cell injection, 3 mice were irradiated with 10 Gy, 3 mice with 8 Gy, and 3 mice with 6 Gy. All irradiated mice showed normal brain architecture and did not have any necrosis or lymphatic damage. It is also interesting to note here that there was a dose-dependent effect of WBI on brain metastatic burden. Images were taken at 20X magnification (50% magnified using Leica’s Aperio ImageScope software.)
B. Combinatorial effects of simvastatin and an optimized whole-brain radiation dose in an IBC brain-metastasis mouse model

First major *in vivo* experiment – 10-Gy single WBI dose plus Simvastatin

**General health of treated mice:** All 70 mice survived through the 5-week period after the 10-Gy radiation treatment. There was significant weight loss and dermatitis in all 40 irradiated mice (20 each in radiation-alone and combination group.) Dermatitis in the field of irradiation was treated with an ophthalmic ointment. Weight loss was managed with gel-pack treatment and mice gained weight slightly after the treatment.

**Tumor burden:** 10 Gy WBI alone significantly reduced the brain metastasis burden compared to no treatment (*p* = 0.0324.) Simvastatin alone (*p* > 0.9999) and the combination of simvastatin and 10 Gy WBI (*p* = 0.1054) did not significantly reduce the brain metastatic burden compared to the control. There was no significant difference in brain metastatic burden between treatment with 10 Gy alone and combination treatment (*p* > 0.9999) [Figure 9]. 4 out of 15 mice (26.67%) in the no-treatment group had close to zero tumor burden. From our lab’s work (13), it is known that the tail-vein-injection brain-metastasis model has a tumor take rate of 67%. In my current experiment, the take rate was 73.33%. Therefore, the number of cells injected was not a problem. Simvastatin appears to increase the tumor burden but it is not significantly different compared to no treatment. The 10-Gy WBI dose was so effective by itself that we might have failed to see any interaction between simvastatin and 10 Gy WBI. This is one of the reasons for changing the dose in my second major *in vivo* experiment.
Table 2: Statistical analysis for tumor burden across no-treatment, simvastatin-alone, 10 Gy WBI–alone and simvastatin–plus–10 Gy WBI treatment groups. Dunn’s multiple comparisons test was used to calculate the p-value for each pair of treatment groups.

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<tr>
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<tr>
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<td>No treatment vs. Statin + 10 Gy</td>
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Figure 9: Tumor burden for the no-treatment, simvastatin-alone, 10 Gy WBI–alone, and simvastatin–plus–10 Gy WBI treatment groups. Tumor burden was calculated by dividing the integrated (top and bottom) GFP pixel area by the integrated total brain area.

Incidence: To determine if there were significant differences in incidence between different treatment groups, the Fisher-Freeman-Halton test was used to
calculate the p-value for each of the three raters separately. All three raters agreed that there was no difference in incidence in any of the four treatment groups. Inter-rater reliability, measured using the Fleiss’ Kappa coefficient for binary categorical measures, was 0.77, indicating substantial agreement between the three raters. The bar graph below shows the incidence of brain metastasis and the corresponding p-values for each rater separately [Figure 10]. All three raters agreed that there was an increase in the incidence of brain metastasis upon treatment with statin alone, but it is not significantly different compared to no treatment.

Rater 1, p=0.49

Rater 2, p=0.69
Figure 10: Brain metastasis incidence for no-treatment, simvastatin-alone, 10 Gy WBI-alone and simvastatin–plus–10 Gy WBI treatment groups. The Fisher-Freeman-Halton test was used to calculate the p-value for each rater separately.

Number of brain metastatic lesions per mouse: Since incidence is a binary measure, we wanted to plot the number of brain metastatic lesions in absolute scale as shown below (Figure 11.) Dunn’s multiple comparisons test was used to calculate the p-value for each pair of treatment groups (Table 3.) The number of brain metastatic lesions was not significantly different between any pair of treatment groups. Though not significant, there was an increase in the number of brain metastatic lesions with statin alone and a decrease in the number of brain metastatic lesions with 10 Gy WBI alone and the combination treatment (10 Gy WBI plus simvastatin) compared to no treatment. Contrary to our hypothesis, simvastatin addition to 10 Gy WBI increases the number of brain metastatic lesions compared to 10 Gy WBI alone. The agreement between three raters for average measure such as the number of brain metastatic lesions was calculated using two-way intraclass correlation coefficient (ICC). The ICC was calculated to be 0.925
with a 95% confidence interval (0.869 – 0.956). An ICC > 0.9 is considered excellent agreement between raters.

Figure 11: Number of brain metastatic lesions for no-treatment, simvastatin-alone, 10 Gy WBI–alone and simvastatin–plus–10 Gy WBI treatment groups. Each dot represents a mouse. Red lines represent the median numbers of brain metastatic lesions in each treatment group.

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Table 3: Statistical analysis for number of brain metastatic lesions per mouse across no-treatment, simvastatin-alone, 10 Gy WBI–alone and simvastatin–plus–10
**Gy WBI treatment groups.** Dunn’s multiple comparisons test was used to calculate the p-value for each pair of treatment groups.

Tumor burden, incidence, and number of brain metastatic lesions in the four treatment groups are represented below (Figure 12.)

![Representative fluorescence–bright field overlay images of IBC3 Brain Metastasis in different treatment groups](image)

**Figure 12:** Representative fluorescence–bright field overlay images of no-treatment, simvastatin-alone, 10 Gy WBI-alone and simvastatin-plus–10 Gy WBI treatment groups. Each of the four images corresponds to the mouse with the tumor burden and number of brain metastatic lesions closest to the arithmetic mean value from its respective treatment group. The bright field image was overlaid with the GFP image to get both the architecture of the brain and its metastatic lesions.
Second major *in vivo* experiment – 9 Gy WBI dose in 3 fractions plus simvastatin:

**Background and hypothesis:**

From the above combination study, it can be seen that a single, high dose of WBI (10 Gy) was so effective by itself that it did not allow statins to sensitize brain metastasis to radiation any further in the combination group. We therefore wanted to reduce the WBI dose and see if statin radiosensitized and reduced the incidence of brain metastasis further in the second major *in vivo* experiment. For the synergistic effects of statin and WBI, we hypothesized that fractionating the dose would give time for tumor cells to repair their damage, resulting in synergistic effects.

In the second *in vivo* experiment, we gave a 9 Gy WBI dose in 3 fractions on days 29, 30 and 31 after cancer cell injection. Also, we chose 9 Gy in 3 fractions because it is a more clinically relevant dose compared to a single dose of 10 Gy WBI while taking cost and logistics into consideration. IBC brain metastasis patients are treated with 30 Gy WBI dose given in 10 fractions, and a 9 Gy in 3 fractions dose is equivalent to 6.2 Gy (assuming the $\alpha/\beta$ value of IBC cells to be 4.5.) The $\alpha/\beta$ ratio describes the ratio of irreparable (alpha) to potentially repairable (beta) DNA damage within the linear-quadratic model of radiation-induced cell killing. The higher the $\alpha/\beta$ value, the better the response of cell line/tumor to fractionated radiotherapy. Figures 8, 9, and 13 shows a dose-dependent reduction of tumor burden and 10 Gy by itself reduced tumor burden significantly. To check the combinatorial effects of statin and radiation, we chose a sub-optimal dose (9
Gy in 3 fractions ~ 6-Gy single dose) for WBI and checked if statins reduced the incidence and brain metastatic burden further when combined with radiation.

**WBI Dose dependent reduction of IBC3 Brain metastasis**

![GFP images taken with Nikon stereomicroscopy.](image)

**Figure 13: WBI dose-dependent reduction of IBC3 brain metastatic burden.** GFP images taken with Nikon stereomicroscopy.

Based on our lab’s previous work (26), I knew that reducing intracellular cholesterol by adding high-density lipoprotein (HDL) radiosensitizes IBC cells *in vitro*. Our lab had found an increase in the initial number of γH2AX foci compared to no treatment when HDL is added, possibly indicating that HDL increased the DNA damage by unknown mechanisms. More importantly, HDL-added IBC cells had persistent γH2AX foci formation, indicative of persistent DNA damage and lack of DNA repair. As simvastatin also reduces intracellular cholesterol by shutting down its synthesis, I hypothesized simvastatin would inhibit the repair mechanisms of IBC cells, which try to clear the damage caused by radiation, resulting in synergistic effects of radiation and simvastatin (Figure 12.) Interestingly, the above hypothesis was supported by a recent paper from another group (27), which showed statins (including simvastatin) delay DNA-repair
mechanisms and cause cellular senescence after irradiation. *In vivo* experiments in the above paper irradiated primary breast tumors and melanoma in mice with pitavastatin. It was not determined whether statins will radiosensitize IBC brain metastasis to whole-brain irradiation.

In the previous experiment, we also gave simvastatin immediately after radiation. We hypothesized that simvastatin may require some time to reach the brain and may not be present immediately when the cells try to repair their damage. To maximize the bioavailability of simvastatin in the brain after radiation, we arbitrarily started simvastatin treatment one week before radiation. All other parameters such as the number of cancer cells used for injection, mode and duration of statin delivery, and so on were kept constant between the first and second experiment.

![Graph showing surviving fraction vs dose](image)

**Figure 14:** Hypothesis for hyper-fractionated low dose WBI for combinatorial effects with simvastatin. The graph was drawn with following $\alpha/\beta$ value – 4.5 for the 9 Gy in 3 fractions and 10-Gy single WBI dose. We hypothesized that with the addition of simvastatin, damage will become increasingly irreplaceable since simvastatin will inhibit
the sub-lethal repair mechanisms after radiation; i.e., the $\alpha/\beta$ value will increase. Simvastatin plus 9 Gy in 3 fractions was drawn assuming the $\alpha/\beta$ value would increase to 7. The linear extrapolation of the curves (see dashed lines), sometimes called the “effective survival curve,” help demonstrate the presence of a shoulder.

**Results for 9 Gy WBI dose in 3 fractions plus simvastatin:**

**Tumor Burden:** The sub-optimal WBI dose, 9 Gy in 3 fractions alone, did not significantly ($p = 0.1378$) reduce the brain metastatic burden compared to no treatment as predicted. But addition of simvastatin to the sub-optimal WBI dose did not radiosensitize and reduce brain metastatic burden significantly compared to radiation alone ($p > 0.9999$) or even no treatment ($p = 0.2519$). This is shows that simvastatin does not radiosensitize IBC3 HER2$^+$ brain metastases to WBI in our model. Interestingly, when simvastatin is given one week before radiation, there is no increase in the brain metastatic burden, which was observed in the previous experiment, compared to no treatment ($p > 0.9999$.) From the no-treatment tumor-burden values (Figure 15), it can be observed that the dynamic range of tumor-burden values across 18 mice was low and many mice had close to zero tumor burden, possible reasons our model is limited in understanding the combinatorial effects of simvastatin and WBI.

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**Table 4:** Statistical analysis for tumor burden across groups of no-treatment, simvastatin alone, 9 Gy WBI dose given in 3 fractions alone, and combination treatment.
Dunn’s multiple comparisons test was used to calculate the p-value for each pair of treatment groups.

![Brain Metastatic burden for Statin plus 3*3Gy experiment](image)

Figure 15: Tumor burden for no-treatment, simvastatin alone, 9 Gy total WBI dose given in 3 fractions alone, and the combination group. Tumor burden was calculated by dividing the integrated (top and bottom) GFP pixel area by the integrated total brain area.

**Brain metastasis Incidence:** To find if there was significant difference in incidence between different treatment groups, the Fisher-Freeman-Halton test was used to calculate the p-value for each of the three raters separately. All three raters agreed that there was no difference in brain metastasis incidence between any of the four treatment groups. Inter-rater reliability, measured using Fleiss’ Kappa coefficient for binary categorical measures, was 0.824 (95% CI 0.690 – 0.957.) Anything over 0.8 is considered
“almost perfect agreement” between three raters. The bar graph below shows the incidence of brain metastasis and corresponding p-values for each rater separately (Figure 16.) All three raters agreed that there was an increase in the incidence of brain metastasis upon treatment with statin alone, but it is not significantly different compared to no treatment. This was observed in the previous experiment as well. Surprisingly, though not significant, there was an increase in incidence of brain metastasis upon treatment with 9 Gy WBI in 3 fractions alone and upon combination treatment compared to no treatment separately. This observation needs further investigation to understand why radiation would increase the incidence of brain metastasis.

Rater 1, $P=0.47$

Rater 2, $P=0.51$
Figure 16: Brain metastasis incidence for no-treatment, simvastatin alone, 9 Gy total WBI dose given in 3 fractions alone, and the combination group. Fisher-Freeman-Halton test was used to calculate the p-value for each rater separately.

Number of Brain metastasis lesions per mouse: Since incidence is a binary measure, we wanted to plot the number of brain metastatic lesions in absolute scale as shown below (Figure 17.) Dunn’s multiple comparisons test was used to calculate the p-value for each pair of treatment groups (Table 5.) The number of brain metastatic lesions were not significantly different between any pairs of treatment group. Though not significant, there was an increase in the number of brain metastatic lesions when mice were treated with statin alone and a decrease in the number of brain metastatic lesions when mice were treated with 9 Gy total WBI dose alone given in 3 fractions of 3 Gy compared to no treatment. The combination of 9 Gy total WBI and simvastatin had almost the same number of brain metastatic lesions compared to no-treatment. Contrary to our hypothesis, simvastatin combined with 9 Gy of WBI increased the number of brain metastatic lesions compared to radiation alone. The agreement
between the three raters for their measured averages was calculated using the two-way intraclass correlation coefficient (ICC). The ICC was calculated to be 0.971 with a 95% confidence interval (CI = 0.957 – 0.981). An ICC > 0.9 is considered excellent agreement between raters.

Figure 17: Number of brain metastatic lesions for groups of no-treatment, simvastatin alone, 9 Gy total WBI dose given in 3 fractions alone, and combination treatment. Each dot represents a mouse. Red lines represent the median number of brain metastatic lesions in each treatment group.
<table>
<thead>
<tr>
<th>Dunn's multiple comparisons test</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment vs. Statin</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>No Treatment vs. 3*3Gy</td>
<td>0.1399</td>
</tr>
<tr>
<td>No Treatment vs. Statin + 3*3Gy</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>3<em>3Gy vs. Statin + 3</em>3Gy</td>
<td>0.8168</td>
</tr>
</tbody>
</table>

**Table 5: Statistical analysis for the number of brain metastatic lesions per mouse across groups of no-treatment, simvastatin alone, 9 Gy total WBI dose given in 3 fractions alone, and combination treatment.** Dunn’s multiple comparisons test was used to calculate the p-value for each pair of treatment groups.
2.5 Discussions and Future Directions

Combining simvastatin with either a fractionated or single WBI dose did not reduce the incidence of brain metastasis. There could be several reasons for this result. First, a single high dose of radiation (10 Gy WBI) almost completely eradicated most brain metastatic lesions, possibly limiting further radiosensitization of brain metastases by statins. A sub-optimal fractionated dose (9 Gy WBI in 3 fractions) did not reduce the tumor burden or incidence of brain metastasis and statins did not radiosensitize them. The dynamic range for the dose of radiation seems to be very small—a high dose is effective and a sub-optimal dose has no effect. It would be interesting to observe what happens to the combination of an 8-Gy single WBI dose with simvastatin or a 12 Gy WBI dose given in 4 fractions with simvastatin.

Second, similar to the finding of Efimova EV et. al, Mol Cancer Ther. 2018 (27), our results did not show any effect of statins themselves on brain metastasis reduction. The radiosensitization by statins in the above paper is dependent on damage caused by radiation; i.e., only radiated samples have synergistic effects with statin. These data also support our hypothesis that statins radiosensitize cells by inhibiting DNA repair mechanism.

Third, though 10 Gy WBI reduced the burden significantly, reducing incidence, i.e. eradicating brain metastasis completely, is a challenging task. Radiation caused substantial cell killing, but leaving even few radio-resistant cells to survive would not reduce the incidence. Radioresistance is major problem in the clinic and our animal model reflects this fact. Because of the difficulty in finding a radiosensitizer, there is
currently no drug approved by the FDA for whole-brain radiosensitizing of brain metastases.

Third, statins prevent plaque formation but have no benefit for patients with high cholesterol deposition in their blood vessels. Statin is therefore known to be a preventative drug in individuals at high risk for atherosclerosis and advanced vascular disease and not very effective for later stages of the disease. My data also suggest that statin has no impact on developed brain metastases (4 weeks after cancer cell injection) but could be effective in the absence of clinically apparent metastatic disease based on our lab’s published data (25). In this investigation, our lab showed that pre-treating cells with statin *in vitro* and then injecting the cancer cells into mice prevented their ability to form metastasis both in orthotropic and experimental tail-vein metastasis models. Injecting SUM149, a triple-negative IBC cell line, *in vivo* through the tail vein first and then treating with simvastatin one week after cancer cell injection reduced metastasis formation. In this paper, simvastatin was shown to upregulate FOXO3a, a tumor suppressor, and have a therapeutic effect. Whether this tumor suppressor has any role in radiosensitization or repair after radiation damage is yet to be explored. These published data suggest that statin is effective in preventing or treating brain metastases at an early stage and not when brain metastases are fully developed, as is the case in my experiments. Simvastatin by itself could be therefore used as a drug to treat breast cancer patients, who are at high risk for forming brain metastases, before they present with clinically apparent metastatic disease.
It is also interesting to note the difference in my experimental results compared to the experiment summarized in Table 1. The previous experiment results (Table 1) show that addition of statins to WBRT decreases metastatic incidence after 10 days of radiation treatment. Assuming the health status of mice had nothing to do with the observed experiment results, these results show that statin delayed the progression of brain metastasis when combined with radiation treatment. This observation can be explained by statin’s role in improved radiosensitivity and reduced proliferation of brain metastatic cells when radiation is given at an earlier time point (21 days) and mice were sacrificed 10 days after radiation treatment. Similar effects of statin do not happen at a later time point like what was observed in my experiments.

1) Improved radiosensitivity: radiation is more likely to kill most of the metastatic cells when there are fewer to begin with. Statins may also be able to improve radiosensitivity to a degree that would allow complete eradication. Since radiation does not kill all cells, over the 5-week period radio-resistant cells would have proliferated, forming the metastatic lesions seen with stereo-microscopy. To find out at what time point there would be no difference in incidence between the radiation-alone and combination-treatment groups, investigators may consider observing what happens between 10 days and 5 weeks after irradiation, i.e. sacrificing mice at the third or fourth week after radiation.

2) Reduced proliferation: statins reduce the fraction of cells entering S phase (25), and thus appear to slow the proliferation of tumor cells. In the previous experiment, the metastatic lesions seen at 10 days after radiation treatment were tiny. It is possible that
statins only slowed cell growth so at that particular time point we saw many fewer metastatic lesions, but after a longer period of time they may have become apparent.

My experiment results are supported by a randomized clinical trial combining simvastatin and WBI in patients with brain metastasis (28). The primary tumors of these brain metastasis patients were either lung or breast cancers. Whether IBC brain metastasis patients were included in their trial within the group of breast cancer patients was not mentioned in the paper, and the trial was not powered to have sufficient numbers of patients surviving until the endpoint of the study. Because of the uncertainty in the statistical results of this clinical trial, we decided to continue testing our hypothesis in our IBC brain metastasis mouse model and obtained results similar to those of the clinical trial, i.e. simvastatin does not radiosensitize brain metastases to WBI.
Chapter 3: Mass spectrometry based tissue imaging to understand the cholesterol distribution and its perturbations by simvastatin in brain metastatic lesions and its microenvironment

3.1 Background:

Mass spectrometer is an important instrument that is used to identify and confirm a biomolecule, or understand structure of a biomolecule etc. Using liquid chromatography coupled with a mass spectrometer (LC-MS), whole proteome and metabolome of different biological samples can be identified. While LC-MS is useful to understand whole proteome level changes of an entire sample, it lacks the ability to capture spatial heterogeneity in distribution of a biomolecule and understand how different cells respond to a certain treatment condition. This limitation is overcome by a technology called Mass Spectrometry Imaging (MSI) which provides spatial information of many biomolecules present at a particular pixel location of a sample.

The specific type of MSI instrument used in this study was a Matrix-Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry (Center for Radiation Oncology Research (CROR) MSI core). This instrument is a high-resolution mass spectrometer specifically configured for imaging biological and chemical materials. Since most abundant compounds detected in a tissue will fall in the range of 50-2000 m/z values, MSI data is typically acquired for this range of m/z values using the mass spectrometer. The detailed procedure of how this technology works is mentioned in the materials and methods section below.
Using the MSI technology, I wanted to understand cholesterol distribution in different cell types present in brain metastasis microenvironment and how simvastatin perturbs this distribution. From the paper (22), I hypothesized that perturbing the cholesterol levels in astrocytes and brain metastatic cells will have therapeutic benefit for IBC brain metastasis. Also, there is no other study which has used MSI to understand whole metabolomic differences between IBC brain metastatic cells and the adjacent cells present in its microenvironment. By overlaying the H&E images with MS images, one could understand the cholesterol distribution in different cell types of brain and heterogeneity in perturbation of simvastatin.

3.2 Materials and Methods

(*- Swaminathan Kumar, **- Richard Larson, ***- Shane Stecklein)

A. Sample Preparation:

Mice was anesthetized and cervical dislocation was performed (S.K.*, R.L.*). Then mice brains were removed (S.K., R.L.) and kept in PBS before imaging it under fluorescent based stereo microscopy (S.K., S.S.*). Brain tissue with GFP positive (IBC3-GFP cells) metastatic lesions were sectioned into slabs using a scalpel, flash frozen with dry ice, and then stored at -80°C until needed (S.K.). Instead of storing the tissue in 10 % formalin, I flash froze the tissue on dry ice without any chemical because formalin or any other chemical can interfere due to its abundance and mask the actual signal in the mass spectrometry imaging data. Using a cryotome, the frozen tissue was cut into 10 μm thickness and 31 such serial sections were placed in 31 glass microscope slides
(Experimental Radiation Oncology (ERO) core). 10 µm was used because it is the ideal thickness for optimized MS parameters for the imaging mass spectrometer instrument. For each tissue, every 5th slide (Slide No. 1, 6, 11, 16, 21, 26, and 31) was stained with Hematoxylin and Eosin (H&E) to verify the presence of brain metastatic lesions (ERO core). Slides with at-least one metastatic lesion were chosen for tissue mass spectrometry based imaging (S.K.). These slides were kept frozen at -80°C until ready for matrix application.

The Shimadzu IM Layer, an automated sublimation matrix applicator was used to coat 2, 5 dihydroxybenzoic acid (for positive mode) on the tissue sections for 30 min (CROR MSI core). The resulting coated slides were subjected to a rehydration step in a heated humidifying chamber for 3 minutes using 1 ml of 9:1 ratio of water and methanol solution respectively (CROR MSI core).

B. Mass Spectrometry Imaging (MSI) procedure

Cryo-sectioned slides were imaged using Waters Synapt G2-Si with Imaging MALDI Mass Spectrometry (CROR MSI core). Imaging of tissue mounted on microscope slides is achieved using a 2.5 KHz NdYAG solid state laser rastered across the tissue sample, giving a chemical composition profile on each corresponding spatial coordinate [Figure 16]. These mass spectral information are collated by the HD Imaging (HDI) software to produce a chemical image that can be correlated to the sample’s histological profile (from H&E staining of adjacent tissue slide) (S.K.).
Prior to loading the coated slide into the mass spectrometer, the slide was scanned using an EPSON scanner for mapping the interested areas into the HDI software (CROR MSI core). The area is selected by the operator visually based on the borders of the brain tissue (CROR MSI core). The laser power was set to 250 (arb units) with 300 laser shots per pixel data (CROR MSI core). The laser raster step was set to 60 µm to match the oval shaped laser spot size of 60 µm (CROR MSI core). Prior to acquiring the data, the instrument was checked for mass accuracy and was calibrated using red phosphorus (36) (CROR MSI core). Since red phosphorus (MW= 30.974) can form clusters of 1-89 such molecules by laser desorption ionization in positive mode, they can be used as a standard to calibrate the machine for mass accuracy up to 3000 m/z values.

**C. Mass Spectrometry Image analysis for cholesterol distribution:**

After acquisition, the HDI software automatically processes the raw data into a collection of images (S.K.). Each image corresponds to a particular m/z ion distribution over the entire tissue. M/z value represents the mass/charge ratio of each molecule which is known from the literature and is stored in the human metabolome database. The software matches each m/z value with a particular molecule of interest by searching the database (S.K.).

Since one of the function of simvastatin is to reduce the cholesterol biosynthesis, I checked for cholesterol distribution across all the four treatment groups. The preferred ion state (more abundant and stable form) of cholesterol {[M+H-H2O]+} was chosen as the m/z value, 369.35 (29), and the corresponding images for each treatment group was
downloaded from the software (S.K.). 3-6 biological replicates (different mice) were imaged for each treatment group (CROR MSI core).

Tumor burden value was calculated as mentioned in chapter 2. Spatial distribution of Cholesterol across the brain parenchyma was observed visually from MSI of the brain slides with metastatic lesions (S.K.). Tumor burden and spatial distribution of cholesterol was compared to see if there was any correlation (S.K.). Cholesterol distribution was not normalized to any standard molecule and the absolute value of cholesterol at each pixel is reported in Figure 17, 18, 19 and 20. The dynamic range of cholesterol was 0-50000, which was kept constant in all the four figures (S.K.).

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**Work flow of MALDI Mass Spectrometry Imaging**

- **Cryo-sectioned tissue slide**
- **Matrix application**
- **Laser ablation by rastering across the tissue sample**
- **Mass spectra collected for each x,y coordinate**
- **Computational analysis using HD Imaging software**

**Figure 18: Mass Spectrometry Imaging work flow.** The frozen tissue slide is coated with 2, 5 dihydroxybenzoic acid for 30 minutes and then 2.5 KHz NdYAG solid state
laser is rastered across the tissue sample to acquire the chemical composition profile on each corresponding spatial coordinate. Mass spectral information is collated by HDI software to produce a chemical image for each m/z ion value that can be correlated to the sample’s histological profile obtained from the adjacent tissue slide stained with H&E.

3.3 Results

A. Cholesterol distribution in brain metastasis and its surrounding microenvironment in the no-treatment group.

The preferred ion state (most abundant and stable state) of cholesterol \([M+H-H_2O]^+\) was chosen as the m/z value, 369.35, and the corresponding chemical image was downloaded for detecting the distribution of cholesterol across the brain sections. The below image [Figure 17] suggests that overall cholesterol distribution across each brain section is low in the no-treatment group compared to radiation treated groups [Figure 19, 20]. From an unpublished microarray data from our group, I found out that the cholesterol biosynthesis pathway genes were upregulated in brain metastasis compared to lung metastasis. So I hypothesized that cholesterol level will be more in untreated brain metastasis but the results were opposite. In the future, the above microarray data and MSI data can be correlated for only after imaging the metastatic lesions of lung tissue. Cholesterol levels were lower in brain metastatic lesions compared to the adjacent normal brain parenchyma visually. We were not able to quantify this observation because the signal of cholesterol is in the noise range (i.e. below 10 \% of highest intensity ion). Ubiquitously present molecules like Phosphatidylcholine was
present above the noise range across all four treatment groups. Also, the tumor burden
did not correlate with the level of cholesterol present in the brain parenchyma or in the
brain metastatic regions.

Figure 19: Cholesterol distribution in the brain parenchyma of no-treatment group
(N=4). The above four brain sections were from four different mice which was not treated.
Scale bar of intensity heat map were kept constant across all four treatment groups
(0-50000) to visually compare the cholesterol distribution.
B. Cholesterol distribution in brain metastasis and its surrounding microenvironment in the statin alone treated group.

Out of five mice which were imaged using mass spectrometry from the statin alone treated group, four of them had low cholesterol level compared to the no-treatment group [Figure 18]. This data suggests that simvastatin inhibited cholesterol biosynthesis (known role of statins) and reduced the cholesterol levels in the entire brain parenchyma as simvastatin is not specific to brain metastatic lesions (entire imaged area). One mouse had more cholesterol levels in the brain parenchyma and could be considered as an outlier. The tumor burden did not correlate with the cholesterol levels in the brain parenchyma or the brain metastatic lesions in the statin alone treated group as well.

Figure 20: Cholesterol distribution in the brain parenchyma of statin alone treated group (N=5). The above five brain sections are from five different mice. Scale bar of
intensity heat map were kept constant across all four treatment groups (0-50000) to visually compare the cholesterol distribution.

C. 10 Gy whole brain irradiation alone and 10 Gy WBI plus statin increases the level of cholesterol distribution in the brain parenchyma.

Three mice brain sections from the 10 Gy radiation alone group [Figure 19] have increased cholesterol levels compared to the no-treatment or the statin alone group [Figure 17, 18]. Six mice [Figure 20] from the combination group (10 Gy radiation plus statin) also have increased cholesterol levels compared to the no-treatment or the statin alone group [Figure 17, 18]. The tumor burden did not correlate with the cholesterol levels in the brain parenchyma or its metastatic regions in both the radiation treated groups.

![Cholesterol distribution in Brain Metastasis treated with 10 Gy alone](image)

Figure 21: Cholesterol distribution in the brain parenchyma of 10 Gy alone treated group (N=3). The above three brain sections are from three different mice. Scale bar of intensity
The heat map were kept constant across all four treatment groups (0-50000) to visually compare the cholesterol distribution.

**Figure 22: Cholesterol distribution in the brain parenchyma of 10 Gy plus statin treated group (N=6).** The above six brain sections are from six different mice. Scale bar of intensity heat map were kept constant across all four treatment groups (0-50000) to visually compare the cholesterol distribution.
3.4 Limitation of above MSI data:

A positive control to confirm cholesterol by using a commercial standard of cholesterol would strengthen the results further. Though one can match the preferred ion state m/z value with the name of the compound by searching through the human metabolome database, the molecule could be confirmed further by spiking in a known standard of cholesterol into the mass spectrometer. Since we used imaging mass spectrometer, the standard would have to be spotted on a slide and then scanned before scanning the actual sample of interest. Though the standard will also be identified by its m/z value, it could be confirmatory because we spiked the known standard manually. Another option is to perform a MS/MS, fragmenting a particular molecule of interest (in my case it is cholesterol) by choosing its m/z value. Unfortunately, the machine used for my studies cannot perform MS/MS of cholesterol to confirm its chemical composition and reconstructing its structure.

Quantification of total cholesterol ion intensity value in the total imaged area of each tissue sample and a statistical test to compare them across different treatment groups would be the ideal way to present the above data. Unfortunately, the total ion intensity value of the imaged area for each tissue falls below the noise range i.e. below 10 % of highest intensity ion. This suggests that the sensitivity of the instrument to measure less abundant molecules is low or the overall cholesterol distribution is very low to be measured by the imaging mass spectrometer used.
3.5 Discussions & Future Directions:

Mass spectrometry based imaging data suggests that even though simvastatin reduced the cholesterol levels in the brain parenchyma, it did not cause any therapeutic benefit for brain metastasis. This is contrary to what was observed in primary glioblastoma cells [22]. Based on this paper [22], we hypothesized that reducing cholesterol levels in the brain metastatic cells could have a therapeutic effect independent of simvastatin role as radiosensitizer. From our data, this does not seem to be the case. Reduction in cholesterol level by simvastatin alone did not have any therapeutic effects such as tumor burden reduction or brain metastasis incidence reduction.

Lack of therapeutic benefits from simvastatin could be due to the compensatory mechanisms from the cholesterol transport pathway. My hypothesis for the compensatory mechanisms is as follows:

When simvastatin reduces cholesterol levels inside the cell, cell senses its low cholesterol level and activates SREBP2 (Sterol regulatory element-binding protein 2) and this transcription factor translocates to the nucleus. SREBP2 transcribes LDL-receptor (LDLR) which pulls the cholesterol from LDL particles outside the cell thus compensating for the cholesterol loss. When there is low cholesterol, SREBP2 also increases HMG-CoA reductase (HMGCR) at the level of transcription. Simvastatin inhibits HMG-CoA reductase enzyme involved in cholesterol biosynthesis. The balance shift towards simvastatin mediated cholesterol reduction or SREBP2 mediated HMGCR increase will decide the net
cholesterol levels inside the cell. While statins are known to reduce blood cholesterol levels in patients with dyslipidemia and prevent atherosclerosis, therapeutic efficacy of simvastatin to reduce brain metastasis seems not to be the case.

The above compensatory mechanisms could be tested at the protein level by immunohistochemistry staining of brain tissue samples to further understand why simvastatin did not have a therapeutic benefit for brain metastasis. Understanding these compensatory mechanisms will help us to identify therapeutic targets that can be combined with simvastatin and WBI to overcome the resistance. If LDLR is upregulated in the tissue samples treated with simvastatin, then we can use a LDLR inhibitor or downstream cholesterol transport inhibitors such as LXR ligand inhibitors (like LXR-623 (22)) to overcome the resistance by triple combination of the inhibitor with simvastatin and WBI.

For unknown reasons at this point, radiation by itself and with addition of simvastatin seems to increase the cholesterol levels in the brain parenchyma. There have been reports of ionization effects of radiation on cholesterol present in the lipid rafts (31, 32, 33). But this needs to be further studied as to how ionizing effect of radiation on the cholesterol would increase the downstream signaling and eventually increase the cholesterol levels in the brain parenchyma.

Even though simvastatin did not radiosensitize IBC brain metastasis, one question that remains to be answered is “Did simvastatin get inside the brain and performed its function of reducing cholesterol”. While my data suggests that simvastatin did reduce
the cholesterol levels in the brain parenchyma in statin alone group using MSI data but this is not true in simvastatin plus radiation group. This result needs to be further validated using two complimentary approaches. – 1). Liquid chromatography mass spectrometry (LC-MS) and western blotting of the whole brain tissue lysates.

Protein lysate from frozen brain tissue samples could be extracted and western blotting for HMGCR, SREBP2, and LDLR could be performed. It would be interesting to know how these proteins are differentially regulated between no-treatment and simvastatin treated samples, and also what happens to these proteins in radiation treated samples. While the western blotting experiment may not be a definitive experiment to know if simvastatin performed its function, still it would help us to understand about the compensatory mechanisms that are acting in the brain once simvastatin is added. At the least, I would expect some change in HMGCR, SREBP2 and LDLR levels between no-treatment and simvastatin alone treated samples to confirm there was some perturbation of cholesterol biosynthesis and transport pathway upon simvastatin addition.

Using LC-MS of brain tissue sample, reduction in mevalonic acid and lanosterol levels in simvastatin treated sample compared to no-treatment could be checked. One could expect HMG-CoA to be high in simvastatin treated sample, since simvastatin prevent HMG-CoA to mevalonic acid conversion by inhibiting HMGCR enzyme. Since simvastatin is not specific to any particular cell type, LC-MS of total brain tissue sample could definitely help us understand if simvastatin worked or not
Ours is the first study (to my knowledge) to generate spatial information of all m/z value from 50-2000 Daltons in brain metastatic regions and surrounding brain parenchyma by correlating MS images with H&E stained images. This study has generated large datasets which could be pursued further to generate hypothesis about the role of sterols and other biomolecules in IBC brain metastasis progression. Region of interest have been drawn around brain metastatic lesions and the adjacent normal brain parenchyma and all the m/z values have been extracted as separate files. Using big data analytic techniques, our lab plans to find differentially regulated molecules between brain metastatic regions and adjacent normal brain parenchyma and validate differentially regulated molecules individually.

Interestingly, when we checked the distribution of many m/z values in the brain using HD Imaging software by eye randomly, we found that some of the m/z values were present only in brain metastatic regions and not anywhere else in the brain parenchyma. These molecules could be studied in detail and could serve as novel imaging bio-markers for identifying brain metastasis or could serve as brain metastasis specific therapeutic targets.
Chapter 4: Overall Conclusions

I tested the hypothesis that simvastatin radiosensitizes MDA-IBC3 brain metastasis to whole brain radiation. I examined both single fraction whole brain radiation (10 Gy) and fractionated whole brain radiation (9 Gy in 3 fractions) with similar results. Simvastatin did not significantly reduce the incidence or burden of brain metastases in this model when radiation was given four weeks after tumor cell injection. Treatment was well-tolerated. Mass spectrometry imaging was performed to determine if simvastatin treatment resulted in observable differences in cholesterol between groups. Preliminary comparison across images suggests simvastatin treatment reduced overall cholesterol levels throughout the brain tissue, however, unexpectedly radiation increased cholesterol levels. Therefore, we were unable to determine whether the desired effect on cholesterol was achieved in irradiated animals. These data serve as pilot information to develop adequate power to investigate this further. If a compensatory increase in cholesterol is caused by whole brain radiation, further exploration of adequate manipulation of cholesterol for radiosensitization may be warranted.

There are several limitations to the work described. First, we have only examined this hypothesis in one animal model. Our lab previously demonstrated in vitro that simvastatin radiosensitizes SUM149 (23), a triple negative (TN) IBC cell line. Among different IBC subtypes, TN IBC (9) and HER2+ IBC (10) have the highest propensity to metastasize to the brain. SUM149 can form brain metastasis through tail vein injection (13). This cell line has BRCA mutation and p53 mutation (34) which could make it sensitive to WBI in vivo since the cells will not be able to repair its damage caused by radiation and
will undergo mitotic catastrophe. Thus, testing simvastatin plus WBI in SUM149 TN-IBC brain metastasis model may be a useful next step to help us understand if the findings are similar to findings in MDA-IBC3.

A second HER2+ model would also add to the robustness of our findings. However, using our tail vein brain metastasis model, none of the other HER2+ IBC cell lines (SUM190 and KPL4) successfully metastasize to the brain (unpublished data from our lab).

An additional limitation is the immunocompromised state inherent to all xenograft models. The SCID-Beige model lacks both T and B cells. Radiation causes death of cancer cells and which in turn releases antigens, some of which are presented by dendritic cells (neo-antigens) to T cells for immune attack against cancer. Since our mouse model lack T cells, it may not mimic what happens in humans. Since we used a human IBC cell line, an immunocompromised mouse was required to prevent cell rejection. There are no syngeneic mouse models derived from IBC cells.

Another limitation is the lack of positive control for radiation sensitization. Unfortunately, there is no proven whole brain radiosensitizer approved in the clinic for IBC brain metastasis which could be used as a positive control. Further, our efforts to assess the efficacy of simvastatin treatment given in the water in the conditions chosen for reduction of cholesterol were limited. Several approaches were undertaken. MSI was used to explicitly assess cholesterol. While descriptive images demonstrate less signal in simvastatin treated versus control no radiation groups, further work is needed to determine if this represents real signal and to power this to analyze significance. Nevertheless, this highlighted a potentially interesting and unexpected finding. The no
radiation brains have lower baseline MSI cholesterol signal than the whole brain irradiated samples. This warrants more rigorous exploration but may imply that in vivo, whole brain radiation may paradoxically upregulate cholesterol beyond that perturbed by simvastatin.

As mentioned in the discussion and introduction before, either simvastatin (25) or PCI (unpublished) were effective by themselves to prevent IBC brain metastasis using our tail vein injection mouse model. Simvastatin and low dose PCI could be tested for its efficacy to prevent the incidence of brain metastasis in our pre-clinical IBC mouse model. If the results are promising, it has a high potential to be translated into the clinic where patients with breast cancer, who are at high risk for developing brain metastasis, can be given very low dose WBRT along with simvastatin thus reducing the toxicity caused by high dose WBRT to the brain.


VITA

Swaminathan Kumar was born in Chennai, Tamil Nadu, India, the son of Kumar Seetharaman and Mangalam Kumar. After completing high school from Zion Matriculation Higher Secondary School, Chennai, Tamil Nadu in 2008, he joined SASTRA University, Thanjavur, Tamil Nadu for his undergraduate studies. He received the degree of Bachelors of Technology in Bioengineering from SASTRA in May 2012. For next three years, he worked as a research assistant in Indian Institute of Science and National Centre for Biological Sciences, Bangalore, Karnataka, India. In August of 2015, he entered The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences to pursue Master of Science degree.

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