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# Therapeutic potential of Rad51 inhibition

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**THERAPEUTIC POTENTIAL OF RAD51 INHIBITION**

by

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# **THERAPEUTIC POTENTIAL OF RAD51 INHIBITION**

**DOUGLAS LE**

## **ABSTRACT**

DNA provides the instructions and regulation of cell growth and survival. Mutations in DNA can cause uncontrolled and unregulated cell proliferation, resulting in cancer. Treatment of cancer involves physical removal of these cells through surgery or inducing cell death by causing irreversible damage to DNA through chemotherapy and radiotherapy. However, natural DNA repair mechanisms may interfere with therapy and may even be increased in cases of therapy resistant cancer. The use of chemotherapy and radiotherapy leads to increased recruitment of DNA repair proteins while aggressive, therapy resistant cancers show overexpression of DNA repair proteins. Rad51 is a protein involved in the homologous recombination (HR) DNA repair process. Rad51 is recruited to sites of DNA damage caused by double stranded breaks, often generated by chemotherapy and radiotherapy. It is expected that inhibition of Rad51 will impair the HR repair process while enhancing the effectiveness of chemotherapy and radiotherapy compared to conventional means. As a result, this literature review aims to identify and examine the drug inhibitors of Rad51 in order to demonstrate the potential viability of this novel treatment in a variety of cancers.

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## ABBREVIATIONS

DNA	Deoxyribonucleic Acid
DNA-PK	Deoxyribonucleic Acid Dependent Protein Kinase
DSB	Double Stranded Deoxyribonucleic Acid Breaks
dsDNA	Double stranded Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor Positive
ESCC	Esophageal Squamous Cell Carcinoma
HDACi	Histone Deacetylase Inhibitor
HeLA	Cervical Cancer Cell Lines
HOS	Human Osteosarcoma Cell lines
HR	Homologous Recombination
IR	Ionizing Radiation
mRNA	Messenger Ribonucleic Acid
NHEJ	Non-Homologous End Joining Repair
NSCLC	Non-Small Cell Lung Cancer
PR	Progesterone Receptor Negative
RNA	Ribonucleic Acid
RNAi	Interference Ribonucleic Acid
RPA	Replication Binding Protein A
SAHA	Suberoylanilide Hydroxamic Acid

SCC	Squamous Cell Carcinoma- Head and Neck
shRNA	Small Hairpin Ribonucleic Acid
siRNA	Silencing Ribonucleic Acid
SSB	Single Stranded Deoxyribonucleic Acid Breaks
ssDNA	Single Stranded Deoxyribonucleic Acid strands
UV	Ultraviolet

## **INTRODUCTION**

### **Cell Cycle Checkpoints**

DNA contains the set of instructions necessary for cellular functions and life. In order for human cells to grow and proliferate, they must progress through several cell cycle checkpoints such as G0, G1, S, G2 and M phase (9). During the G0 phase, the cell is stable and not actively dividing. In the G1 phase, the cell is growing and prepares and organizes DNA for duplication. S phase results in DNA duplication. G2 leads to reorganization of the cell. M phase refers to mitosis, which results in cell division and the formation of new cells. DNA damage will prevent the cell from advancing to the next checkpoint and will result in cell death through apoptosis or triggering DNA repair processes, potentially resulting in cell survival (12). As a result, the cell cycle checkpoints act as regulation for the growth and proliferation of new cells along with regulation of cell survival. These checkpoints have great implications for cancer.

### **Cell Cycle Dysregulation**

Cancer is the result of uncontrolled cell growth and division. Cells become cancerous when the cell cycle checkpoint is ignored and growth of these cells becomes unchecked and unregulated (12). Normal checkpoint mechanisms are ineffective and damaged, mutated, or defective DNA is allowed to pass. This can cause abnormal cell function or result in further mutations potentially causing greater proliferation of the cancerous cells (12). Apoptosis does not activate and the cells can form into a tumor or

cause accumulation of abnormal cells within the blood. This dysregulation results in pathology and can compromise the organs along with the host.

### **Accumulated Cell Damage**

Although cancerous cells can ignore cell cycle arrest signals even with DNA mutation or DNA damage, other apoptosis mechanisms can be triggered in the event of significant cell stress or DNA damage (15). Inducement of cell stress or DNA damage can overwhelm the ability of the cancerous cell to grow and proliferate by interfering with the cell machinery. This interference can be due to interaction with critical cell growth materials such as, DNA, RNA, or proteins, or due to interaction with signal transduction (15). The end goal is to generate significant damage to the cancerous cell in order to prevent it from functioning and proliferating. As a result, cancer therapies take advantage of this method through the use of chemotherapy or radiotherapy.

### **Improving Effectiveness of Cancer Therapies**

Chemotherapy and radiotherapy interfere with cell proliferation through induction of DNA damage, thus preventing the cell from replicating (15). While this may stop cancerous cells from growing and induce apoptosis, natural repair mechanisms may render it less effective. Inducing DNA damage through the formation of DNA double strand breaks (DSB) from ionizing radiation (IR) results in the recruitment of DNA Homologous Recombination (HR) repair mediators such as the Rad51 protein (27). Since the goal of chemotherapy and radiotherapy is to cause significant DNA damage, the

ability of cancerous cells to repair DNA damage would be expected to decrease the effectiveness of these types of therapies. As a result, direct or indirect suppression of DNA repair mechanisms, such as inhibition of Rad51, may be a valid avenue for improving these conventional therapies.

Inhibition of Rad51 has shown interesting results and promise in the improvement of cancer therapies. A study using antisense oligonucleotides, complementary sequences used to inhibit DNA, resulted in inhibition of cell proliferation and a 90% increase in radiosensitivity correlated with 99% decrease in levels of Rad51 mRNA and 90% decrease in Rad51 protein levels in normal mouse cells (35). This demonstrated a powerful mechanism for Rad51 inhibition and set the stage for testing in cancerous cells. However, it raised the question of how Rad51 inhibition would perform in cancerous cells and live animal models.

The authors, Ohnishi and Taki, repeated the Rad51 antisense inhibition experiments using mouse glioma cells in culture and in an animal model while using normal neurons as controls (36). Their experiment revealed that in-vitro antisense inhibition of Rad51 decreased mRNA levels by 90% and protein levels by 70% in glioma cells, improved in-vitro and in-vivo radiation sensitivity by decreasing tumor survival, and improved survival rates in mice treated with radiation compared to control (36). These results on mRNA inhibition were similar to the last study but showed that antisense inhibition may not be as effective in practice. Despite these mixed but promising results, there was interest in finding out how Rad51 inhibition affected normal mouse neurons after being exposed to radiotherapy.

Most interestingly, the antisense oligonucleotides were tagged with florescent markers, which indicated limited visibility around normal tissue while the glioma cells became highly florescent. This implied that cancerous cells expressed more Rad51 compared to normal cells and that normal tissues were spared from radiosensitizing effects. At that time, the mechanisms were unclear as to why normal tissues were spared but the authors stated that Rad51 levels were highest during G2 and S phase, proliferative phases of the cell cycle, which could have affected expression of Rad51 levels (36). This suggested that Rad51 activity has specific preferences based on the cell cycle. While these studies used antisense oligonucleotides as Rad51 inhibitors, there was a need to find drugs that targeted this process.

It was discovered by Slupianek et al. that increased Rad51 levels and DSB repair were moderated by an oncogenic tyrosine kinase known as BCR/ABL in leukemia cell lines (31). The high levels of Rad51 and increased activity of DSB in those leukemia cells contributed to decreased efficacy and resistance to chemotherapy drugs such as cisplatin and mitomycin C (29). As a result, the authors then repeated their experiment using Gleevec, an ABL tyrosine kinase inhibitor used in the treatment of leukemia. The experiment revealed that Gleevec decreased Rad51 levels in leukemia cells while restoring efficacy to cisplatin and mitomycin C (29). Consequently, Gleevec was tested in other types of cancers such as human gliomas cells.

Gleevec was used on 2 different human glioma cell lines which resulted in decreased Rad51 mRNA and protein levels, prevented recruitment of Rad51 to sites of DSB caused by radiotherapy, and increased rates of radiotherapy-induced apoptosis while

sparing regular human fibroblast cell lines from these effects (29). These results using Gleevec with human glioma cells were similar to the antisense in-vitro and in-vivo experiments using mouse glioma cells. Both improved radiotherapy while sparing normal cells from the potentially damaging effects of radiosensitization by inhibiting HR repair.

### **Specific Aims/Objectives**

Since these experiments have demonstrated promising results using novel techniques to improve chemotherapy and radiotherapy, the goal of this paper is to review primary literature in order to identify different mechanisms and methods to suppress RAD51, a protein involved in the recruitment of homologous recombination (HR) DNA repair machinery, highlight differences and inconsistencies with study design and analysis, demonstrate why inhibition of Rad51 is an appropriate target for the improvement of cancer therapies as a first line treatment, and suggest future directions for study. It is expected that decreasing the ability of the cell to repair DNA damage through Rad51 inhibition will render it more susceptible to radiotherapy and chemotherapy, thus improving the effectiveness of conventional treatment. This paper will focus on reviewing literature from similar studies to determine if the use of Rad51 inhibitors improves conventional therapeutics such as radiation therapy or chemotherapy.

## **CANCER THERAPIES AND THE ROLE OF DNA REPAIR**

### **Conventional Cancer Treatment**

The usual avenues for treating cancer rely on four major types of treatment: surgery, radiotherapy, chemotherapy, or combination therapy. Surgery is based on physical removal of the tumor or cancerous cells. Radiotherapy involves the use of radiation to damage DNA and induce cell death. Ionizing radiation damages DNA by causing single stranded DNA breaks (SSB), double stranded DNA breaks (DSB), and DNA lesions, all of which can prevent cell growth, proliferation or cause cell death (41). Chemotherapy uses a molecular method to generate alkylation, addition of alkyl groups to DNA, causing DNA damage and cell death. Alkylating agents can damage DNA by causing base damage, lesions, formation of bulky adducts, crosslinking, along with SSB and DSB (16). These methods cause DNA damage, resulting in inhibition of growth of rapidly dividing and proliferating cells and can induce apoptosis. Treatment using chemotherapy or radiotherapy generally causes DSB (8). While surgery, radiotherapy and chemotherapy are effective methods for treating cancer, a combination of these types of therapy or multimodal therapy has been demonstrated to be more effective in treating a wide variety of cancers. However, multimodal therapy is not well tolerated in older populations or patients with certain health conditions. As a result, it is necessary to identify methods that will increase the effectiveness of radiotherapy or chemotherapy while minimizing dosage or exposure to these therapies. Focusing on DNA repair mechanisms will provide new avenues for improvement.



## **DNA Repair**

DNA repair has 2 main repair mechanisms, Non-Homologous End Joining Repair (NHEJ) and Homologous Recombination Repair (HR). NHEJ works at all phases of the cell cycle but is mostly active in the G1 phase while HR prefers the G2/S phases (8). Focusing on HR repair is important for improving cancer therapies. This is due to the fact that HR repairs DSB generated by chemotherapy and radiotherapy (16,17). There is evidence to show that radiotherapy and chemotherapy specifically trigger HR repair which causes recruitment of proteins involved with DNA repair. For example radiotherapy causes increased phosphorylation of histone H2AX, indicating DSB, while activating HR repair through increasing the expression and recruitment of Rad51 protein to the sites of DSB (27). This test was used in many studies to identify the presence of DNA damage and repair. Chemotherapy can also generate DSB but through different pharmacological mechanisms. As expected, there was increased HR repair and expression of Rad51 following chemotherapy (1). Since chemotherapy and radiotherapy are based on inflicting DNA damage through formation of DSB and HR is activated by these DSB, impairing HR through inhibition of Rad51 expression or association should increase the effectiveness of these modalities.

## RATIONALE FOR TARGETING RAD51

Inhibiting Rad51 and the HR repair process represents a potential and reasonable mechanism for targeted therapy. Rad51 protein serves as a critical part of the HR repair process. Rad51 protein is recruited to the nucleus and binds to sites of DSB in order to repair naturally accumulated damage or DSB caused by chemical, UV, or IR methods (14, 32). After induction of DSB, the damaged strands are separated into two single stranded DNA strands (ssDNA) which allows for Replication binding protein A (RPA) to bind (40). RPA is an ssDNA binding protein that serves as a dock for Rad52, a protein that stimulates and interacts with Rad51 and RPA (40). This allows for Rad51 to make contact with the ssDNA strands since it has specificity for double stranded DNA (dsDNA) without RPA (22). After Rad51 forms a nucleoprotein filament on the tail of the ssDNA strand, it participates in strand exchange with homologous dsDNA (40). This process is repeated with the other ssDNA strands, allowing the tails to serve as primers for DNA repair (**Figure 1**) (40). While it may be problematic to inhibit repair of naturally occurring DNA damage and possibly cause malignant mutations, the benefits of Rad51 inhibition in cancer cells far outweigh the risk. This is due to the fact that Rad51 inhibition provides the benefit of increasing therapeutic efficacy, overexpression of Rad51 leads to poorer outcomes, and Rad51 overexpression plays a critical role in metastasis.

As mentioned in the introduction, the experiments using siRNA targeting mice glioma and Gleevec targeting human glioma cells demonstrated improved sensitivity to

radiation therapy and chemotherapy respectively. These experiments demonstrated the feasibility of targeting Rad51. Targeting Rad51 is further supported by numerous studies that have indicated that Rad51 may be overexpressed in cancerous cells and leads to poor outcomes for many different types of cancers such as colorectal, esophageal squamous cell carcinoma, cervical, and breast cancer.

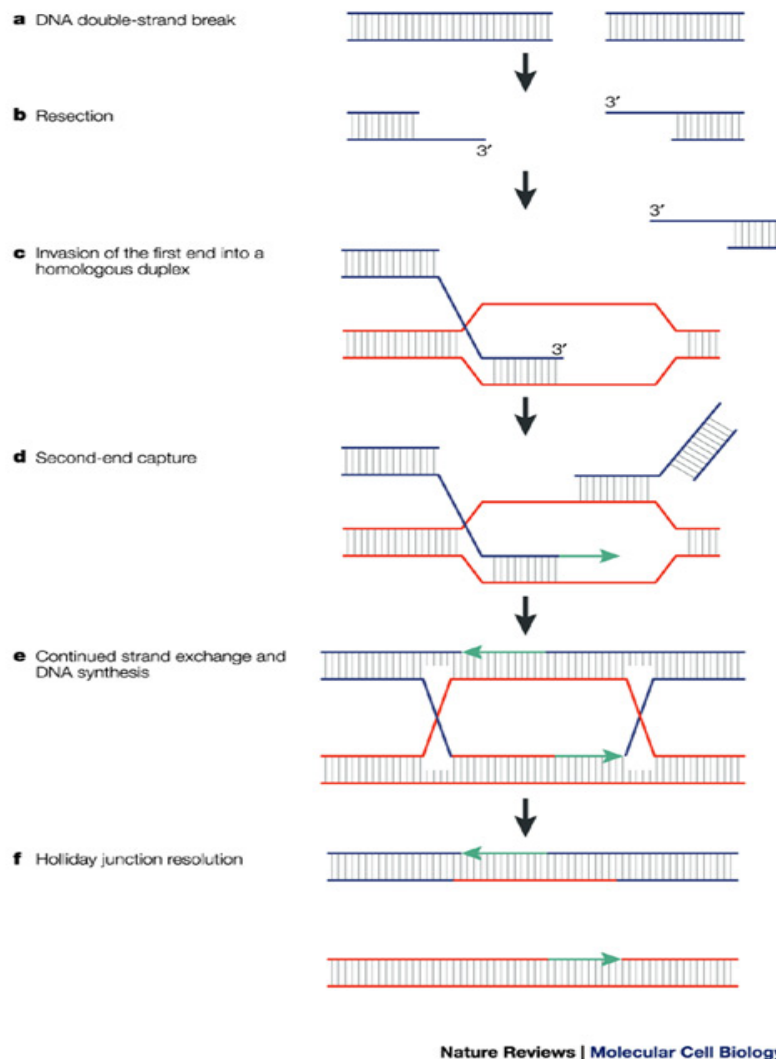


Figure 1. **Rad51 Repair of Double Stranded DNA Break (DSB).** (Figure taken from West, et al., 2003) (40)

A. DSB

B. Resection of DSB exposes two single stranded DNA (ssDNA) tails, which allows binding of replication protein A (RPA), a dock for Rad52 binding. Interaction with Rad52 allows Rad51 to bind the ssDNA-RPA unit.

C. Rad51 forms a filament on the ssDNA tail, allowing the complex to participate in strand exchange with homologous double stranded DNA (dsDNA).

D. The second ssDNA tail is recruited by Rad51 and/or Rad52.

E. The tails serve as DNA primers for synthesis.

F. Completed crossovers result in separation of the strands.

## **Different Cancers Show Elevated Rad51**

An immunohistochemistry analysis of 1200 colorectal cancers revealed that 54% of the biopsies were negative for Rad51 expression while the remaining 46% were categorized as weak (34%), moderate (11%), or strong (1%). The authors found that overall survival was significantly correlated with Rad51 expression ( $p=0.001$ ), with median survival at 76 months for weak expression, 46 months for moderate, and 11 months for strong (37). They also found that Rad51 expression was significantly correlated as an independent prognostic test ( $p = 0.011$ ) along with predicting tumor stage and status ( $p < 0.0001$ ) (37). These data indicate that increased Rad51 expression played an important role in overall survival, could be used as a prognostic marker, and targeted drug therapy might provide value to these patients.

Analysis of post-surgical resection of 230 esophageal squamous cell carcinoma (ESCC) patients showed that nearly half of the patients (46.5%) showed high expression of Rad51 and these patients had statistically significant ( $p=0.034$ ) decreased overall survival compared to those with low expression (61.6 months vs. 70.2 months) along with statistically significant ( $p = 0.031$ ) decreased disease free survival (58.4 months vs 67.7 months) (19). These data also indicated that increased Rad51 expression is correlated with poor outcomes compared to patients with decreased Rad51 expression. The data also showed that Rad51 was a statistically significant ( $p = 0.021$  and  $p = 0.013$ ) independent prognostic marker for overall survival and disease free survival (19). These results for ESCC are similar with colorectal cancers in correlating Rad51 expression with prognosis and patient survival.

Patients with cervical cancer may also benefit from Rad51 inhibition. Two groups of 21 undergoing mRNA study and 24 patients undergoing histological study with advanced cervical cancer were treated with combined chemoradiotherapy and selected for biopsy. Half of these patients showed complete remission while the others had partial response. Patients with incomplete response to therapy showed significantly elevated levels of Rad51 mRNA and nuclear protein in the genomics group ( $p = 0.016$ ) and histological group ( $p < 0.0001$ ) (2). This study also showed that BRCA1, a protein that localizes with Rad51 and initiates DSB repair, was significantly elevated for patients with incomplete response in the genomics group ( $p = 0.032$ ) and histological group ( $p < 0.0001$ ) (2). Elevation of Rad51 and BRCA1 levels appear to be prognostic indicators for whether or not a patient will have complete remission following treatment. As a result, cervical cancer patients with elevated levels of Rad51 would likely benefit from targeted inhibition.

High levels of Rad51 were also discovered in breast cancer and significantly correlated with poorer outcomes. A study which examined biopsies from 75 patients found that high levels of Rad51 significantly correlated with estrogen receptor positive (ER) / progesterone receptor negative (PR) tumors ( $P = 0.03$ ) and these types of tumors were identifiable on immunohistochemical analysis ( $p = 0.003$ ) (4). Taken alone, these findings indicated that Rad51 expression and breast cancer subtype could be identified by histological examination by using Rad51 protein as a marker. But the authors also analyzed the microarray RNA from 295 patients and found that high levels of Rad51 led to elevated risk of relapse ( $p = 0.015$ ), metastases ( $p = 0.009$ ), and poorer survival ( $p =$

0.013) while the PR positive subtype resulted in better prognosis ( $p = 0.015$ ) (4). It is interesting to note that this study indicated that elevated levels of Rad51 may lead to metastasis. However, the mechanism was not identified. Combined with these data, histological analysis of Rad51 as a diagnostic marker could be a potential method for identifying specific breast cancer subtype that is likely to have poorer outcomes and would benefit from certain types of therapies, including Rad51 inhibition.

### **Influence of Rad51 on Therapeutic Efficacy and Metastasis**

While the previous studies have correlated Rad51 overexpression with poorer outcomes, there is also indication that it may have a role in metastasis. A study on esophageal squamous cell carcinoma (ESCC) examined the influence of Rad51 on 89 patients who were treated with surgery and 39 patients who received neoadjuvant chemoradiotherapy, their first exposure, followed by surgery. The surgery only patients with high levels of Rad51 had significantly greater frequency of metastasis ( $p = 0.0168$ , 58% vs. 30%) compared to those with low levels of Rad51 (23). Since these cells have not been treated and therefore accumulated therapy induced DSBs, it is likely that the elevation in Rad51 contributed to permitting cell survival in cells that have genetic irregularity. However, the mechanism for how this could lead to metastasis was not clarified in this article. Similar to other studies, elevated Rad51 led to significantly decreased frequency of therapeutic response for patients receiving neoadjuvant chemoradiotherapy compared to those with low expression of Rad51 ( $p = 0.0171$ , 46.5% vs. 68.8%) (23). Clearly, Rad51 overexpression reduced therapeutic efficacy for

chemoradiotherapy while those without overexpression showed better responsiveness. For these data, it is possible that Rad51 inhibition could result with a 20% increase in combined chemoradiotherapy efficacy and decreased frequency for metastasis in patients with ESCC by half.

While previously mentioned studies demonstrated the correlation between Rad51 overexpression and metastasis, the following study by Wiegman et al. provided crucial insight on the mechanism for Rad51 on metastasis (42). Their immunohistochemistry analysis of 235 breast cancer tumor samples indicated that Rad51 overexpression correlated with metastatic status and high tumor grade along with identifying a mechanism by which Rad51 supports metastasis. Triple negative breast cancer tissue showed twice the amount of Rad51 protein compared to normal tissue (**Figure 2**) (42). Invasive ductal carcinoma showed 3 times the frequency of Rad51 overexpression compared to invasive lobular carcinoma ( $p=0.0008$ , 26% vs. 9%) (**Figure 2**) (42). Tumor progression data from 23 patients were matched to progression of ductal carcinoma in-situ to lymph node metastasis showed that increasing tumor severity correlated with higher frequency of Rad51 overexpression (**Figure 2**) (42). Patients matched to progression of invasive lobular carcinoma to lymph node metastasis and primary invasive ductal carcinoma to brain metastasis also showed the same correlation (**Figure 2**) (42). These data reveal that certain subsets of breast carcinomas are more prone to Rad51 overexpression, which correlated with increased severity and frequency of metastasizing.

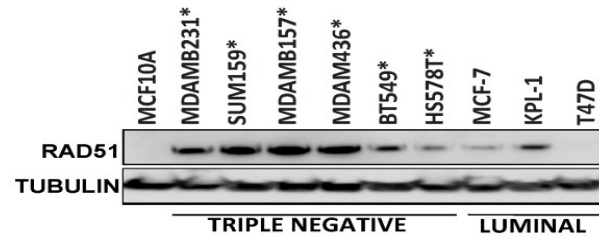
In order to determine the role of Rad51 on metastasis, 4T1.2 cells, a type of mammary carcinoma prone to metastasis in mice, were implanted in vivo and Rad51 was



silenced using small hairpin RNA (shRNA). Use of Rad51 silencing in 4T1.2 cells resulted with significantly decreased tumor growth rate ( $p=0.0013$ ) and metastasis compared to controls not receiving shRNA (**Figure 3C**) (42). This provided evidence that Rad51 demonstrated great influence over tumor progression. However, Rad51 inhibition through shRNA decreased tumor growth rate and decreased maximum volume by a fourth, highlighting the great benefit of targeting this mechanism and possibly including it with conventional therapies.

This experiment was repeated following resection of the primary tumors and Rad51 silenced mice showed delay in primary tumor regrowth while control mice showed metastasis of secondary tumors (**Figure 3D**) (42). Both experiments with shRNA indicated that Rad51 overexpression is responsible for faster tumor growth and required for metastasis to occur. This is critical since the ability of inhibiting Rad51 could stop this type of aggressive progression.

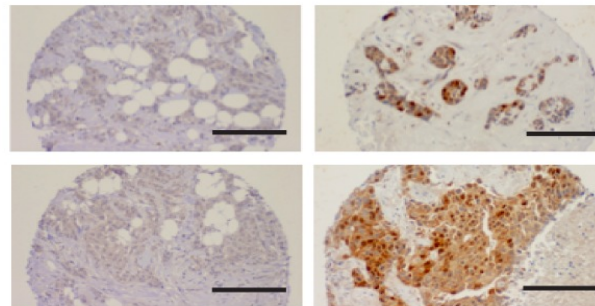
**A.**



**B.**

Primary tumour data	total	RAD51 +ve	%	P value
Subtype				
IDC	121	32	26.4%	0.0008
ILC	114	10	8.8%	
Tumour progression data				
DCIS/LCIS	23	0	0%	0.0031
IDC/ILC	23	4	22%	
LN Met	23	9	44%	
ILC	114	10	8.8%	0.0470
ILC LN metastasis	28	7	25%	
IDC primary	39	8	20%	0.0512
IDC brain metastasis	39	17	43%	

**C.**



### Figure 2. Rad51 Expression and Tumor Progression

(Figure taken from Wiegman, et al., 2014) (42)

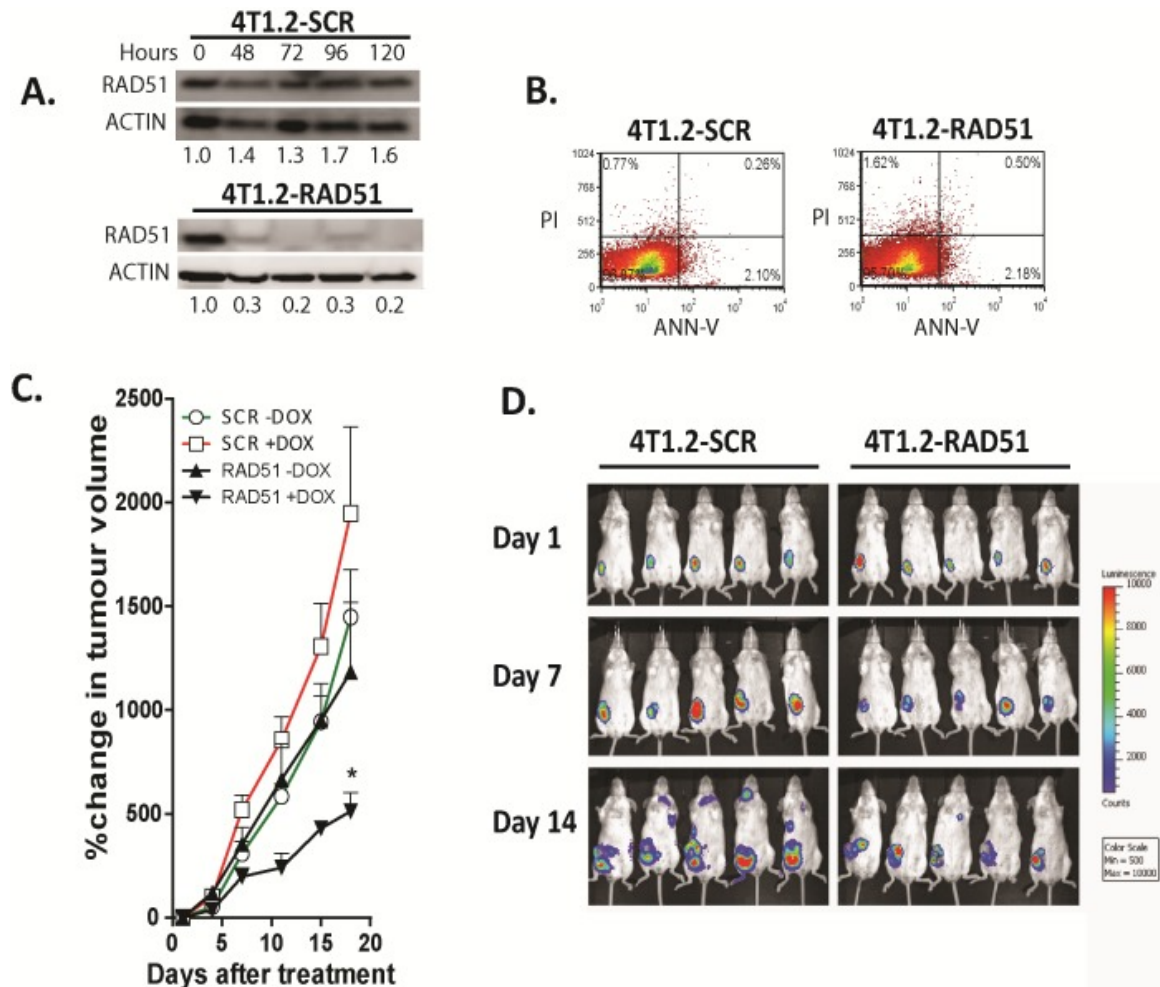
A. Triple negative tumors marked with asterisk compared to unmarked normal tissue. Shows increased Rad51 expression in triple negative breast cancers.

B. Immunohistochemistry microarray of 235 tumors demonstrating increased frequency of Rad51 overexpression correlating with tumor severity.

C. Histological Evaluation of Rad51 Expression

Left: Primary breast carcinoma

Right: Metastatic carcinoma showing increased Rad51 expression



**Figure 3. Relief of Metastatic Effects from Rad51 Silencing in 4T1.2 Breast Carcinoma Cells**

(Figure taken from Wiegman, et al., 2014) (42)

- Exposure to Doxycycline triggers shRNA silencing of Rad51 after 48 hours in 4T1.2 carcinoma cells compared to control.
- Both types of cells were viable after 7 days
- Indication that that shRNA induced by Doxycycline resulted with decreased rate of tumor growth
- Comparison of Rad51 silenced 4T1.2 to control revealed massively decreased occurrence of distal metastasis.

The question of how Rad51 overexpression is responsible for metastasis can be answered by a different experiment using metastatic human breast cancer cells, MDA-MB-231. Implantation of MDA-MB-231 cells into mouse lungs showed that the mice treated with shRNA did not display in metastasis while over half of the control mice had metastasis in distal organs (**Figure 4**) (42). It appeared that Rad51 was required for implantation and colonization at distal sites. Wiegman et al. analyzed Rad51 silenced cells and found that morphology indicated that although there was a metastatic-like change from mesenchymal to epithelial, there were no changes in stem cell markers or cell adhesion proteins required for colonization (42). They confirmed these data by repeating the experiment with Rad51 overexpressed BT549 breast cancer cells which resulted in metastasis and increased expression of stem cell markers and cell adhesion proteins (42). These interesting results indicate that Rad51 overexpression is responsible for molecular and structural transformations that allow the carcinoma to colonize and metastasize in distal organs while silenced cells prevent this effect. Genetic analysis of Rad51 silenced cells showed decreased expression of genes responsible for metastatic proliferation and invasion along with decreased expression of genes that play a role in regulation of mammary gland development, such as c/EBP $\beta$  (42). This study on breast cancer metastasis has been crucial in identifying the role that Rad51 plays in metastatic tumor implantation and colonization, morphological changes that support distal metastasis, and genetic influence on dysregulation of normal growth processes. Hopefully this evidence will spawn further study and analysis.

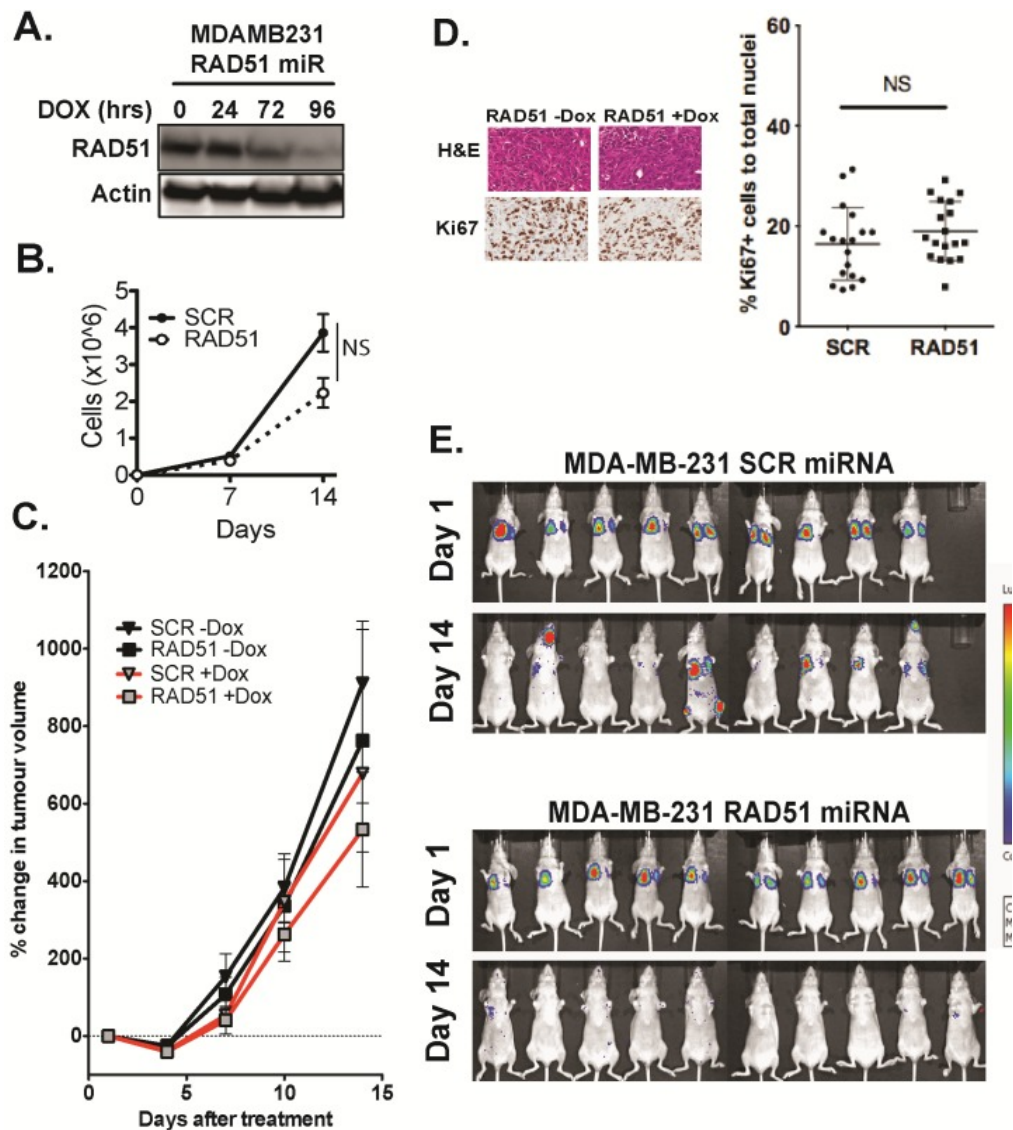


Figure 4. Requirement of Rad51 for Metastasis in MDA-MB-231 Human Breast Carcinoma Cells

(Figure taken from Wiegmans, et al., 2014) (42)

- Exposure to Doxycycline triggers shRNA silencing of Rad51 after 24 hours.
- In vitro analysis of cell growth and viability between Rad51 knockout and control showed lack of significant effect.
- Rad51 silenced cells showed decreased rate of tumor growth compared to control.
- Rad51 silenced cells show no distal metastasis compared to over 50% metastasis in control after day 14.

The rationale for targeting Rad51 has been highlighted by studies which indicated that Rad51 influenced therapy outcome by affecting therapeutic efficacy, it has been overexpressed in a wide variety of tumor types compared to normal cells, and it influences metastatic progression and lethality. Influence on therapeutic efficacy was demonstrated with Rad51 overexpression and activation of HR repair at sites of DSB caused by therapeutic intervention. Studies using siRNA or pharmacological intervention provided evidence of increased therapeutic efficacy and apoptotic activity through Rad51 inhibition. Since it was demonstrated that Rad51 was overexpressed in many types of cancers while not overexpressed in regular cells, this provided the basis for safely targeting cancer specific cells while sparing healthy cells. The fact that these studies also showed statistically significant correlation of Rad51 overexpression with disease progression and utility as a prognostic marker highlight the value of performing more studies analyzing the role of Rad51. Most importantly, Rad51 overexpression has been shown to be required for metastatic progression and significantly correlated with increased frequency of metastasis and cellular changes required for metastasis. These factors combined provide logical evidence for targeting Rad51 as a means to improve therapeutic efficacy and clinical outcomes in cancer patients. As a result, the remainder of the paper will focus on literature reviews of published studies to in order to determine which Rad51 inhibitors exist and the performance of these inhibitors in various in vitro and in vivo models along with highlighting areas of concerns and future directions.

## **Literature Review: Viability of Rad51 Inhibitors**

### **Published Studies: Existing Chemotherapeutic Inhibitors**

After performing a literature review, there appear to be recent discoveries on the mechanisms of many existing chemotherapeutic interventions. These mechanisms have been identified as direct or indirect inhibitors of Rad51 function. Since it was understood that Rad51 inhibition may increase sensitivity to chemotherapy and radiotherapy along with preventing or halting metastasis, these agents were tested in a wide variety of cancers and models to determine efficacy. The results were very promising. There are also new experimental inhibitors that directly target Rad51 and these results will be summed as well. Most interestingly, researchers may have discovered a way to deliver targeted gene therapy to inhibit Rad51 only in carcinomas while sparing normally functioning cells. This may result in greater benefit compared to pharmacological delivery.

### **Data**

#### **Erlotinib**

Erlotinib is a tyrosine kinase inhibitor of EGFR, epidermal growth factor receptor, which has shown to be upregulated in head and neck squamous cell carcinoma (SCC) and non-small cell lung cancer (NSCLC) and leads to poorer outcomes, decreased therapeutic

efficacy, and radiation resistance (7). Studies showed that EGFR mediates signaling of DNA dependent protein kinase (DNA-PK), which is required for activation of HR and NHEJ DSB repair (3). This study examined Erlotinib treatment alone, x-ray therapy alone or combination of Erlotinib and X-ray therapy in in-vitro and in-vivo models for human NSCLC, prostate cancer cells, and human head-and-neck SCC.

In-vitro use of Erlotinib showed inhibition of radiotherapy induced activation of EGFR, inhibited expression of Rad51 protein, increased radiosensitivity and decreased carcinoma cell survival (7). Tests using Human Non-Small Cell Lung Carcinoma (H226) and Human head-and-neck Squamous Cell Carcinoma (UM-SCC1) showed that Erlotinib and radiation combined resulted in a 35% to 75% increase in apoptosis compared to Erlotinib alone (10% to 25%) or radiation alone (25% to 50%) (7). This increase was additive, which demonstrated the strength of combined therapy compared to monotherapy (7). Erlotinib prevented activation of EGFR receptor after radiation exposure, potentially minimizing the risk of radiation-induced regrowth of cancer cells (7). Erlotinib successfully prevented an increase in Rad51 expression after radiation exposure while irradiated cells showed elevated Rad51 expression (7). After exposure to increasing doses of radiation, Erlotinib moderately increased radiation response by decreasing cell survival by 10% at all radiation doses compared to controls (7).

In-vivo study of these tumors in mouse xenographs revealed that combination therapy was superior to Erlotinib or x-ray therapy alone and histological analysis favored combination therapy (7). Further studies using in-vivo xenographs of both cell lines showed that after 60 days, Erlotinib alone decreased tumor volume by 20%, radiation



therapy alone decreased tumor volume by 50% compared to controls, and Erlotinib + radiation therapy was superior at decreasing tumor volume by 75% with statistical significance (7). Histological study of in-vivo lung cell carcinoma growth showed that combination therapy demonstrated the least staining of tumor proliferation markers and EGFR markers, monotherapy groups (radiation alone or Erlotinib alone) showed moderate levels of staining, and untreated controls showed the most amount of staining (7).

### **Imatinib**

Imatinib inhibits several tyrosine kinases but most importantly it inhibits c-ABL, a regulator for increasing Rad51 expression and formation around DSB (8). In-vitro studies were performed on normal fibroblast (GM05757), prostate adenocarcinoma (PC3), pancreatic adenocarcinoma (PANC1), bladder carcinoma (RT112M), and human lung carcinoma (H1299-DR-GFP) cell lines. Evidence showed a 40 to 50 percent decrease in Rad51 expression, 60 to 70 percent decrease in HR repair efficacy, and decreased association of Rad51 at the sites of DSB that were independent of the cell cycle (8). Interestingly there was only a 30 to 40 percent decrease in HR repair efficacy with siRNA (8). In-vitro studies demonstrated that multimodal therapy was more effective in causing mitotic catastrophe than monotherapy due to increased chemosensitivity and radiosensitivity along with decreased cell survival in tumors but not in fibroblasts (9). The in-vivo study using mouse prostate adenocarcinoma xenografts showed great clinical practicality for using Imatinib combined with radiation. Those results showed that Imatinib with radiotherapy improved the delay in tumor growth after

exposure to radiation compared to controls using radiation alone or Imatinib alone (8). Tests of mice weight and post-mortem histological study of the gut lining showed that weight and gut toxicity were comparable to irradiated controls (8).

### **Panobinostat**

Panobinostat is a histone deacetylase inhibitor (HDACi) that suppresses tumor growth by modifying chromatin access along with affecting transcription, signaling, and DNA repair proteins (13). The author's goal was to examine the role of Panobinostat on HR repair proteins on muscle invasive bladder cancer cells (RT112) in combination with radiotherapy and with radiotherapy alone. They found that nanomolar amounts of Panobinostat were effective for downregulation of Rad51 and other HR repair signaling proteins such as MRE11 and NSB without affecting Ku70 or Ku80, NHEJ repair proteins (13). Controls did not show downregulation. Compared to untreated cells, Panobinostat increased tumor kill rates and decreased Rad51 protein levels, which were found to correlate with delayed  $\gamma$ H2AX foci formation following ionizing radiation therapy (13).

### **SAHA**

Suberoylanilide Hydroxamic Acid (SAHA) is another HDACi that is approved for treatment of T-cell lymphoma by impairing HR repair through modification of chromatin structure (6). The change in histone modification prevents Rad51 from accessing DSB and affects signaling processes independent of the cell cycle (6). These experiments demonstrated decreased cell survival following radiotherapy, increased and sustained  $\gamma$ H2AX foci, and resulted in a 3-fold decrease in Rad51 levels along with reduced Rad51

binding to  $\gamma$ H2AX foci. The massive decrease in functional Rad51 protein and prevention of Rad51 binding to sites of DSB are likely to be responsible for the decreased survival which were also seen in tests using human multiple myeloma cell lines (MM1.s, U226B1, RPMI8226, KMS-11) when combined with exposure to radiotherapy (Figure 5B) (page 25) (6).

There is clear indication that SAHA + radiotherapy is superior to monotherapy radiation. Cyclin A levels, a marker for G2/S cycles which are preferred by Rad51, remained consistent between controls and SAHA treated cells in all 4 cell lines which indicated that changes in Rad51 expression were not due to effects on the cell cycle (6). The authors provided further evidence showing that SAHA specifically inhibited HR repair through Rad51 depletion and by preventing Rad51 from associating with chromatin, which was indicated by increased histone acetylation (Figure 5A) (page 25) (6).

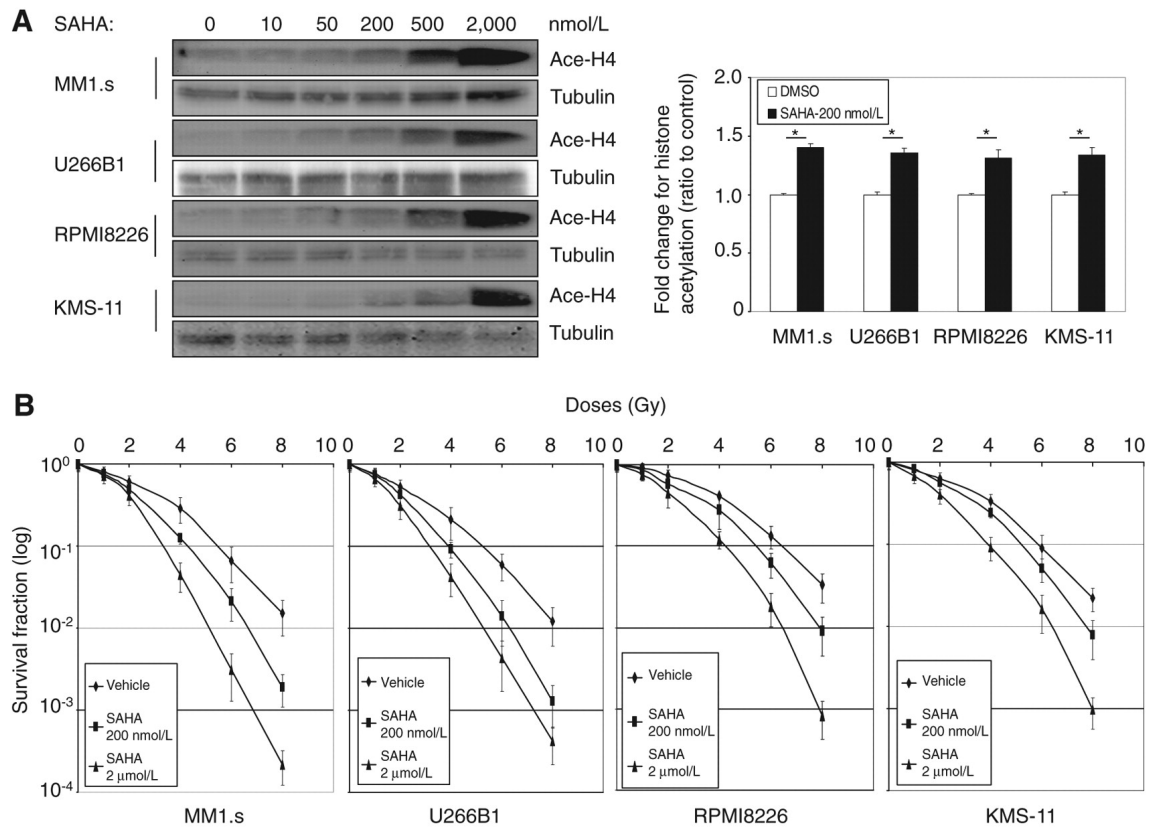


Figure 5. Chromatin Accessibility To Rad51 Influences Radiation Sensitivity. (Figure taken from Chen, et al, 2012) (6).

A-Left: Increasing dosage of SAHA increases histone H4 acetylation. Right-Acetylation increases histone folding compared to controls.

B-SAHA increases radiation sensitivity at increasing radiation doses through decreasing cancer cell survival.

## **Methotrexate**

While previous articles either focused on depletion of Rad51 protein or inhibition of Rad51 assembly at sites of DSBs, use of a DNA synthesis inhibitor also decreased Rad51 formation and function. Methotrexate is a DNA synthesis inhibitor currently used in the therapy of various cancers such as acute lymphoblastic leukemia and osteosarcoma (11). The purpose of this study was to identify the mechanism between use of Methotrexate and inhibition of HR repair in Human Osteosarcoma Cell lines (HOS). Methotrexate is known as an inhibitor of dihydrofolate reductase, which decreases the amount of tetrahydrofolate required for DNA synthesis (11). Data from this study showed that Methotrexate decreased Rad51 mRNA transcripts which reduced Rad51 protein levels thus preventing repair of DSBs through inhibition of HR repair (11).

Rather than use  $\gamma$ H2AX as a marker for unrepaired DSBs as in previous studies, the authors used comet assays to quantify the amount of DNA damage. Compared to HOS cells exposed only to radiation, Methotrexate in combination with ionizing radiation (IR) produced longer comet moments, indicating that there was twice the amount of DNA damage (11). Using a HR Repair Assay, an I-SceI endonuclease to induce DSBs, the authors showed that there was inverse correlation between length of comet moments and DSB repair efficiency (11).

This raised the question of whether or not inhibition of DNA synthesis was the reason for this decrease in efficiency or if Methotrexate directly affected HR repair through Rad51. Testing showed that there was no antibody staining of Rad51 protein in Methotrexate treated with IR and there was a 100% decrease in Rad51 mRNA but lower

than 20% decrease in BRCA2 and Rad52 mRNA (11). NHEJ deficient HOS cells treated with increasing doses of Methotrexate showed vastly decreasing rates of colony formation with a maximum of twice the magnitude compared to wild type HOS cells (11).

## **Experimental Rad51 Inhibitors**

### **IBR-2**

Mutations in upstream factors such as Bcr-abl, which is responsible for Rad51 overexpression, can decrease Imatinib efficacy (44). As a result, the authors designed a study to identify small molecule inhibitors of Rad51 protein that would be effective against Imatinib resistant cancer. Their experimentation resulted in the discovery of IBR-2, a small molecular inhibitor that binds directly to Rad51 protein. The molecule was designed with similarity to BRCA2 binding motifs (BRC motifs), a factor which Rad51 binds to. The results revealed important and critical data. IBR-2 targeted a structure known as 1524-FHTASGK-1530 in order to prevent Rad51 protein from associating with BRC motifs, resulting in an attack on specific assembly sites (44).

The authors used an I-SecI GFP to introduce DSBs. IBR-2 decreased the fraction of GFP positive cells, IBR-2 + ionizing radiation (IR) decreased Rad51 foci by half and greatly decreased colony survival with increasing doses of IR (44). Mechanistic study of IBR-2 action revealed interesting data. IBR-2 did not affect Rad51 mRNA levels but specifically decreased Rad51 protein levels (44). Other proteins, such as Rad50 and ERK,

showed normal results indicating that IBR-2 specifically interfered with Rad51 protein formation which led to increased breakdown of Rad51 protein through increased proteasome activity (44). Use of a proteasome inhibitor proved this hypothesis correct since it resulted in increased Rad51 protein stability (44).

Interference with Rad51 protein function and accelerated proteasome action also resulted with inhibition of cancer cell growth and increased cell death in many different cell lines such as human leukemia (K562), metastatic human breast cancer (MDA-MB-231), triple negative human breast cancer (MDA-MB-468), estrogen responsive human breast cancer (MCF7), human ductal breast cancer (T47D), and human breast epithelial cells (HBL100) (44). Efficacy after 24 hours showed double the efficacy, which was attributed to accelerated proteasome activity (44).

These results suggested that IBR-2 might show efficacy against in-vivo xenographs of mice using the Chronic Myeloid Leukemia cell lines (T315I) which are resistant to Imatinib and its second-generation tyrosine kinase inhibitors, Dasatinib and Nilotinib. IBR-2 decreased tumor growth and volume by 75% compared to control without side effects (44). Using Rad51 RNAi as an inhibitor to test for IBR-2 specificity showed similar outcomes. Tests of tolerability showed that IBR-2 did not significantly affect normal cord blood cells or bone marrow cells while decreasing multidrug resistant Chronic Myeloid Leukemia cells (K562) by a third (44). Combined with Imatinib, these effects on blood and bone marrow remained consistent but decreased leukemia cells by two-thirds (44). Further analysis showed increasing rates of cell death correlated with increasing doses of IBR-2 and decreasing levels of Rad51 protein (44). Protein levels of

Bcl-xL remained consistent, indicating that IBR-2 did not induce cell death through this anti-apoptotic regulator (44).

### **Gene therapy**

This following study by Cao, et al., explored gene therapy to directly target a toxin to a specific Rad51 promoter, Rad51C. This novel experimental therapy involved the viral delivery of diphtheria toxin A in order to decrease protein levels of Rad51C. Comparison of normal cell lines to 4 breast cancer cells HCC1954, MCF-7, T47D, and MDA-MB-231, cervical cancer (HeLa), kidney cancer (GP2-293), and fibrosarcoma (HT1080) showed that cancer cells had 6 times the amount of Rad51C mRNA and 10 times the amount of Rad51C protein while other proteins such as Rad51B and Rad51D did not show statistically significant elevation (5). Cloning a fragment of the promoter with luciferase or GFP visually demonstrated that these fragments specifically targeted cancer cells and these cancer cells showed a 200-fold increase in Rad51C activity (5). Gene therapy results showed that the promoter fusion of Rad51C to diphtheria toxin A decreased cancer cell survival down to about 10% without significantly affecting regular cells which showed 90% survival (5). Similar to the varying levels of Rad51C expression, Rad51C levels decreased up to 10 times with variance between different cancer cell lines but did not significantly affect regular cell lines (5). Although this technique was effective at reducing levels of Rad51C and decreasing survival, it was not effective for all cancer lines. Breast cancer cell line (HCC1954) had 50% survival while HeLa and GP-293 breast cancer lines had 20% survival (5).



## **Discussion**

### **Erlotinib**

The study of Erlotinib on head and neck squamous cell carcinoma (SCC) and non-small cell lung cancer (NSCLC) indicated that epidermal growth factor receptors (EGFR) and EGFR inhibitors play important roles in cancer therapy. The in-vitro studies showed that radiotherapy alone was most effective for treating both SCC and NSCLC since it resulted with the lowest cell survival. However, addition of Erlotinib to radiotherapy decreased cell survival in an additive manner, which supported Erlotinib's role as a radiosensitizer. This radiosensitizing effect was supported by the fact that Erlotinib prevented radiotherapy induced Rad51 expression, indicating that Erlotinib achieved its effect by preventing an elevation in this HR repair protein. Exposure to different doses of radiation showed that Erlotinib moderately improved radiation response by a maximum of 40%. A 40% improvement in radiation response applied to conventional radiotherapy could significantly improve patient outcomes and survival rates. Not to mention, this may allow for more tolerable radiation dosing regimens by decreasing the dose applied but still maintaining high efficacy.

Improvements in outcomes and survival were supported and demonstrated by mouse xenograph studies. These in-vivo studies showed that radiation therapy was most effective at decreasing tumor volume by a half but also provided evidence showing that Erlotinib with radiation therapy maximized tumor shrinkage by 75%.

Immunofluorescence studies of in-vivo tissue showed that Erlotinib with radiation

therapy was superior at suppressing tumor proliferation and EGFR markers compared to monotherapy or controls. Tumor shrinkage and suppression of these markers provided strong evidence to support in-vitro studies but also demonstrated that the results were not just due to action on Rad51.

There were some weaknesses in this study that decrease the ability to interpret the link between Rad51 expression and treatment outcomes. For example, there was a lack of Rad51 protein quantification. Quantifying levels of Rad51 protein would allow for analysis of statistical correlation to radiosensitization, tumor survival, and tumor volume. Although this study did not identify the mechanism between upstream inhibition of EGFR receptors and downstream effects on Rad51 expression, a recent study indicated that Erlotinib inhibition of EGFR receptors suppressed downstream Mek/Erk and AKT kinase pathways which prevented the interaction of insulin receptor substrate 1 (IRS1) to Rad51 thereby inhibiting HR repair (39).

Despite those weaknesses, the in-vitro and in-vivo studies highlight the importance Rad51 suppression and its role on radiosensitization, tumor survival, and tumor shrinkage. The study also supported the addition of Erlotinib to conventional radiotherapy due to improved efficacy and therapeutic response. All of these factors could lead to improved patient survival and outcomes. Overall, the effect of Erlotinib on Rad51 suppression support combination therapy and further studies into Rad51 inhibitors.

## **Imatinib**

Similar to Erlotinib, Imatinib is also a tyrosine kinase inhibitor. The difference is that Imatinib inhibits c-ABL, which is responsible for increased Rad51 expression and is upregulated in certain cancers. Use of Imatinib in prostate adenocarcinoma, pancreatic adenocarcinoma, bladder carcinoma, and human lung carcinoma showed that decreasing Rad51 protein by half limited HR repair to 30% of normal function. Tests of cell survival showed that combination therapy such as Imatinib with radiation or Imatinib with chemotherapy (Mitomycin C or Gemcitabine) was significantly more effective in decreasing cell survival than monotherapy without affecting regular cells. While Erlotinib increased apoptosis, decreased cell survival by Imatinib was due to mitotic catastrophe. In-vivo studies using pancreatic adenocarcinoma indicated that Imatinib with radiation was superior at increasing the delay in tumor growth compared to monotherapy without changes in gut toxicity.

The quantification of Rad51 protein levels and its relationship to HR repair efficacy is quite useful for determining how much Rad51 inhibition can improve conventional therapies. In-vitro studies provided statistically significant data favoring the use of Imatinib with either radiation or chemotherapy rather than use these conventional therapies alone or without the action of Imatinib on Rad51 levels. It is important to highlight that Imatinib decreased tumor survival without significantly affecting the normal fibroblast cells. This suggests that Imatinib has the ability to improve response without impairing or harming regular tissue. In-vivo prostate adenocarcinoma xenographs supported this claim since mice exposed to Imatinib and radiation had comparable weight

and gut tissue to mice that were exposed to radiation only. Although in-vivo data did not indicate the magnitude of Imatinib as a radiosensitizer or how much it could affect tumor volume like the Erlotinib study, the data does support use of Imatinib with radiation since it significantly delayed the return of tumor to its previous untreated volume. Reduced tumor growth may allow for clinicians to use different strategies for treating cancer. Other studies could be performed to analyze the ability of Imatinib to shrink tumor volumes compared to other therapies along with using different tissue xenographs in order to compare efficacy.

Most interestingly, the data indicated that use of siRNA for Rad51 protein showed that HR repair was maximally functional at 70% while Imatinib showed maximum efficacy at 30%. It appeared that Imatinib was much more effective than siRNA targeting. This is quite unlike the mouse glioma experiments, which demonstrated much higher efficacy using antisense oligonucleotides. It would be of interest to see if siRNA could be another way to inhibit Rad51 levels and to test how it affects HR repair efficacy and chemo or radiosensitivity in other cell lines. Other interesting data showed that Imatinib decreased cell survival through mitotic catastrophe while Erlotinib decreased cell survival through increased apoptosis. Nature Reviews indicated that mitotic catastrophe is part of a non-apoptotic pathway (26). Although both are tyrosine kinase inhibitors with different targets and show a shared similarity in decreasing Rad51 function, there is no clear indication why use of these drugs led to different cell death pathways. Further analysis identifying this mechanism could lead to improved targeting of cancer cells.

Regardless of these interesting data generated by the study, analysis of the majority of the study indicated that Imatinib showed great applicability in a wide variety of cancer types. These different types of cancers responded well to chemotherapy and radiotherapy upon inhibition of Rad51 protein levels. In-vivo studies showed delayed tumor growth and comparable gut toxicity, which could aid health professionals in developing treatment plans along with improving prognosis and quality of life. Overall, the data supported the use of Imatinib and inhibition of Rad51 as a chemosensitizer and radiosensitizer with a favorable safety profile.

### **Panobinostat**

While Erlotinib and Imatinib targeted tyrosine kinase activity to affect downstream mediators of Rad51 expression, Panobinostat is a histone deacetylase inhibitor (HDACi) that interferes with the ability of Rad51 to bind to sites of DSBs by affecting its access to chromatin by modifying histone properties. Results showed that 50 nanomolar and greater doses of Panobinostat alone downregulated Rad51 and HR repair signaling proteins in bladder carcinoma cell lines, decreasing cell survival down to about 20%. Rad51 knockout lines showed greater survival than bladder carcinoma lines, indicating that interfering with chromatin access affected factors other than Rad51. Identification of this mechanism may allow for improved targeting and drug design to take advantage of decreased tumor survival. Interestingly NHEJ deficient cell lines were most affected by Panobinostat. This indicates that patients with NHEJ deficient bladder cancers would benefit the most from Rad51 inhibition or Panobinostat.

Tests of radiosensitivity showed that 25 nM doses of Panobinostat with radiation decreased cell survival to around 10%, which was comparable to Rad51 knockout lines while NHEJ deficient lines showed less than 10% survival. Delayed formation of  $\gamma$ H2AX foci indicated that Panobinostat increased DSBs and delayed its repair. Compared to controls, these results indicated that use of Panobinostat and inhibition of Rad51 function doubled the effectiveness of radiotherapy. Being able to double the effectiveness of radiotherapy might benefit patients by allowing more tolerable radiation doses or regimens, possibly decreasing tissue damage and risk of creating new malignancies. This could improve access for older patients since treatment guidelines for bladder carcinoma call for an initial dose of chemotherapy followed by surgery, radiotherapy or a combination of chemo- and radiotherapy, and both surgery and radiotherapy have similar results and are not well tolerated (18).

Although this study demonstrated the effect of Panobinostat as a radiosensitizer, there was no indication whether or not it would also hold true as a chemosensitizer as seen from the Imatinib study. It would be interesting to perform an analysis of whether Panobinostat is a better radiosensitizer or chemosensitizer. Quantification of Rad51 protein levels, HR repair assays, and histone folding could also provide deeper insight as to the degree of changes required to have an overall impact on cell survival. This data could be compared to other HDACi and other types of cancers with upregulated histone deacetylase activity in order to determine how to produce the best outcomes. Additionally, it would have been interesting to see how Panobinostat affects in-vivo bladder carcinoma xenographs and whether or not it would be tolerable. These data could

reveal great insights into the applicability of Panobinostat and histone modification as a means to inhibit Rad51 and improve conventional therapeutics. In conclusion, this study was the first to identify the role of Panobinostat as a radiosensitizer in bladder carcinomas through inhibition of Rad51 and HR repair function by novel means of histone modification.

## **SAHA**

Another HDACi that has shown efficacy in some studies is Suberoylanilide Hydroxamic Acid (SAHA). SAHA is used for certain cancers such as T-cell lymphoma. Further review of this drug revealed that changes in chromatin induced by targeting histones correlated a threefold decrease in Rad51 protein with decreased cell survival. This is supported by data from Figure 5B (page 32) which showed that increased acetylation increased histone folding, leading to increased cell death compared to untreated controls at all radiation doses. Although levels of Rad51 protein were decreased, this histone modification also prevented the remaining Rad51 proteins from accessing the DSBs. This effect was seen and consistent in 4 different human multiple myeloma cell lines. While HR repair assay data showed that SAHA specifically targeted HR repair, it appeared that siRNA was more effective at inhibiting HR repair. These results are opposite of the Imatinib study, indicating that it might be due to cancer specific differences or due to targeting different mechanisms. Other studies could be performed with identical cell lines, siRNA inhibitors of Rad51, and same medication, Imatinib and/or SAHA, to identify which method is the most efficacious. Overall, these

data demonstrated the mechanism and radiosensitizing effects of SAHA and provide support for its use in convention therapies.

Although the study demonstrated significant evidence supporting the use of radiotherapy combined with SAHA as a Rad51 inhibitor, the authors did not test the effect of Rad51 inhibition of chemotherapeutic agents. Chemotherapeutic agents can also induce DSB and it would be interesting if future studies tested and compared different therapy regiments: chemotherapy alone, radiotherapy alone, chemotherapy and radiotherapy combined, chemotherapy and SAHA combined, radiotherapy and SAHA combined, or a combination of all 3 treatments. The ability for SAHA to inhibit HR repair and radiosensitize should yield similar results with other techniques that can introduce DSB. Other studies such as the Imatinib review mentioned previously have indicated that inhibition of Rad51 mediated HR repair does result with increased chemosensitivity. However, these studies have not examined the combination of combined chemoradiotherapy verses combined chemoradiotherapy + Rad51 inhibitor. Such studies may reveal which is the most appropriate for clinical use and best patient outcomes. Future clinical studies should also test to see if SAHA reduced toxicity can be used to decrease radiotherapy or chemotherapy doses while maintaining high efficacy. This may be a more efficient and effective method for improving patient outcomes, quality of life, and 5-year survival rates.

### **Methotrexate**

A study designed to understand how Methotrexate interfered with HR repair in



osteosarcoma revealed the unusual link between tetrahydrofolate depletion and specific depletion of Rad51 mRNA. HR repair assay data showed that Methotrexate doubled radiation induced DNA damage and decreased repair efficacy by half compared to controls. These Methotrexate treated lines showed complete lack of antibody staining for Rad51 protein, indicating that Methotrexate was very successful at inhibiting Rad51 function and prevented the radiation induced production of Rad51 protein which lowers therapeutic efficacy and increases risk of metastasis. Although Methotrexate is a DNA synthesis inhibitor, quantification of Rad51 mRNA levels showed a 100% decrease but did not significantly affect other mRNA transcripts such as BRCA2 or Rad52. This data revealed the odd novelty of Rad51 protein levels being linked with DNA synthesis, raising the question of why Rad51 was specifically targeted yet other mRNA transcripts were not. Identifying this link could lead to better methods of inhibiting Rad51 function along with potentially targeting other proteins linked to cancer progression. The fact that this data showed decreased colony formation from Methotrexate treated cells supports the role of Rad51 inhibition in decreasing metastasis.

Future studies could be done to improve analysis of this study and other previously reviewed studies. For example, analysis of Methotrexate action on cell cycle distribution could illuminate whether or not Rad51 depletion was due to a decreased fraction of cells in S phase. As mentioned earlier, Rad51 is highly active in the G1/S phase and the S phase is likely targeted by Methotrexate. However, this alone does not explain the data showing that other mRNA transcripts were relatively unaffected. Other experiments could be performed for deeper analysis of Methotrexate inhibition of HR

repair by comparing radiotherapy cell survival of treated lines to untreated lines. This data could quantify the link between HR repair efficacy, DNA damage, and cell survival. Cell survival assays using untreated controls, treated normal cells, and treated malignant cells might indicate best dosage for targeting Rad51 without adversely decreasing other mRNA transcripts. Combined with further radiosensitivity and additional chemosensitivity tests could reveal optimal strategies for combating cancer proliferation along with yielding valuable data for future therapies. To summarize, this study of Methotrexate applied to human osteosarcoma cell lines has revealed the novel link between inhibition of DNA synthesis and targeted Rad51 depletion, generating more questions and avenues to explore in improving cancer therapeutics.

### **Experimental Rad51 Inhibitors**

Although Erlotinib, Imatinib, Panobinostat, SAHA, and Methotrexate are currently used therapeutics that have recently been discovered to affect upstream and downstream factors involved in Rad51 protein levels, researchers have now turned their focus onto experimentally targeting Rad51 function. One of these is an experimental drug, IBR-2, and the other involves gene therapy.

### **IBR-2**

IBR-2 is a drug designed to overcome Imatinib resistance due to overexpression of Bcr-abl, the target of Imatinib, which directly contributes to Rad51 overexpression. As a result, targeting Rad51 protein was necessary since Rad51 overexpression leads to adverse effects such as resistance to chemotherapy and radiotherapy, increased tumor

survival, and metastasis. Data from the study showed that IBR-2 alone was successful at inhibiting HR repair and IBR-2 with radiation doubled the efficacy of radiotherapy, which adversely affected colony survival. These survival results were similar to Methotrexate but not as potent as the cell survival results from Erlotinib or Panobinostat but are difficult to directly compare due to use of different cell lines.

Analysis of Rad51 levels showed that Rad51 mRNA was not affected while Rad51 protein levels were decreased and the protein was prevented from interacting with DSBs. Dose response studies provided inverse correlation between IBR-2 and Rad51 protein levels. Data showed that other protein levels were unaffected, indicating Rad51 specific targeting, which could reduce side effects on in-vivo studies along with providing clearer interpretation of Rad51 interaction. Use of a proteasome inhibitor showed the novel effect of how Rad51 proteins undergo accelerated breakdown due to IBR-2 inhibition of Rad51 protein assembly. It is unclear if other drugs that prevent Rad51 assembly or association with DSBs also undergo accelerated proteasome breakdown. Since Panobinostat and SAHA also prevent Rad51 association with DSBs, addition of a proteasome inhibitor and protein analysis to these studies could indicate whether or not this effect was due to IBR-2 action or due to prevention of Rad51 association with DSBs. Taking advantage of this mechanism could prevent the radiation induced increase of Rad51 levels seen after radiotherapy exposure.

In-vitro studies on leukemia and 4 different aggressive breast cancer cell lines showed that this Rad51 inhibitor was successful at increasing cell death, reducing growth, and could be used in a wide variety of cancers. Due to Rad51 specific targeting, the

authors conducted in-vivo studies and wide variety of tolerability tests using Chronic Myeloid Leukemia lines, which are Imatinib resistant. The in-vivo tests showed highly successful results, with both IBR-2 and Rad51 RNAi decreasing tumor volume by 75%. Tests comparing IBR-2 to Rad51 RNAi are critical for evaluating the effectiveness of IBR-2 inhibition of Rad51 and these tests showed that the results were equivalent. It was interesting to see that IBR-2 with Imatinib decreased cell survival by an additional third, indicating that IBR-2 overcame Imatinib resistance and rendered the drug useful again.

This combination of IBR-2 with Imatinib highlighted the idea that a Rad51 inhibitor by itself, although highly effective in this scenario, might demonstrate greater effectiveness with other therapeutics that target Rad51 and/or other signaling pathways. Although the authors indicated that increasing doses of IBR-2 correlated with decreased Rad51 protein, there were no indications of whether or not IBR-2 with Imatinib decreased Rad51 even further. These results would be necessary for determining if the mechanism for overcoming Imatinib resistance was due to other factors, such as signaling, or due to greater impairment of Rad51 function. These data could allow researchers to determine if complete suppression of Rad51 protein is necessary or if targeting multiple Rad51 pathways would lead to improved efficacy.

Further tests indicated high tolerability without side effects since IBR-2 did not affect normal blood cells, bone marrow cells, or mice weight. Other reviewed studies used normal fibroblasts, mice weight, or histological examination of gut tissue to test for toxicity. The use of normal blood cells and bone marrow cells appeared to be unique compared to the other studies and provided valuable data for the next step in research.

Future tests involving Rad51 inhibition as a chemosensitizer or radiosensitizer should include normal hematological cells as controls for tolerability testing along with the commonly use fibroblasts. These data would be useful for in-vivo studies and human application should it reach that stage.

These results, along with radiosensitivity data, support the use of IBR-2 as a supplement to improve conventional therapies. Overall the data suggest that IBR-2 was successful at improving chemotherapy and radiotherapy response in a variety of cancer types and was highly effective at overcoming in-vivo drug resistance without side effects, thus providing support for future studies in other types of cancers.

### **Experimental Gene Therapy**

The experimental gene therapy study provided novel data that may lead to improved Rad51 targeting. This study showed that a specific promoter, Rad51C, showed between 6 to 10 times elevation of Rad51C mRNA or protein in 4 different breast cancer lines, cervical cancer, kidney cancer, and fibrosarcoma. This study was the first to indicate this subtype of Rad51 is significantly elevated in multiple types of cancers, thereby providing a potentially useful target. The use of a diphtheria toxin A poison fused to this Rad51C promoter in all of these cell lines resulted in higher efficacy than Erlotinib, Imatinib, Panobinostat, SAHA, Methotrexate, or IBR-2 by only allowing on average, 10% cancer cell survival yet allowing 90% of normal cells to remain viable. These results demonstrated the potential of targeting a more specific subtype of Rad51 protein that is unusually elevated in a wide variety of cancers, many of which were the

same lines used in those other studies.

However effective this therapy appeared, one breast cancer line showed 50% survival while cervical and another breast cancer line showed 20% survival. These data demonstrate that although Rad51C may have the broadest applicability in wide varieties of cancers, certain cancer subtypes show some individual variance to Rad51C targeting. This was similar to discrepancies within the other reviewed studies. Future studies of gene therapy and other drugs that target Rad51 should test for effect in more cancer lines and cancer subtypes to determine if Rad51 targeting has an even broader applicability than demonstrated. Considering that this study was very novel, it is possible that therapies designed to inhibit Rad51C protein may vastly improve its use with conventional chemotherapy or radiotherapy. The ability to deliver a poison to Rad51C elevated cancer cells combined with a general Rad51 inhibitor and conventional therapies may future decrease cancer survival to below 10%, which could result with massively improved outcomes and cell survival. It may even be advantageous to examine whether or not other highly elevated proteins in cancers are viable targets for this type of gene therapy. In conclusion, this gene therapy study provided valuable evidence for a new method of attacking cancer cells along with potentially improving conventional therapies.

## **Conclusion**

The data from studies of HR repair suggested that targeting this process could lead to efficacy improvements in cancer therapies. Investigation into that line of reasoning revealed that Rad51 was highly elevated in multiple types of cancers and cancer subtypes, encompassing cervical carcinoma, many breast cancers, esophageal squamous cell carcinoma, lung cancer, colorectal cancer, and many hematological malignancies. Early studies showed that suppressing Rad51 function greatly improved radiosensitivity and chemosensitivity. Numerous studies to determine the value of Rad51 expression as a prognostic indicator showed that overexpression of Rad51 was usually seen in patients with aggressive carcinomas. In-vivo studies by Wiegman, et al., using different cancer lines, provided crucial data indicating that Rad51 greatly influenced tumor growth and progression and was required for metastasis to distal organs (42).

As a result of the mounting evidence showing the role of Rad51 in cancer, many authors revisited commonly used therapeutics with the goal of determining whether or not Rad51 was a critical part of the mechanism. Their data revealed that the radiosensitizing effects caused by Erlotinib, Imatinib, Panobinostat, SAHA, and Methotrexate were due to impairment of Homologous Recombination repair through interference with Rad51 function. Although it was critical to identify the role of Rad51 in decreasing cancer growth and progression, these studies also provided valuable data showing that suppression of Rad51 function could vastly improve chemotherapy and radiotherapy efficacy in a vast variety of cancers.

From these successful results, other authors started focusing on ways to target Rad51 function more efficiently by performing screening for inhibitors of this protein. This led to an experimental inhibitor known as IBR-2 to directly target the protein and inhibit its assembly. The data provided evidence that the drug was successful at Rad51 inhibition in many types of blood cancers. What was most interesting was that it even overcame therapeutic resistance and improved therapeutic response with other chemotherapeutics. Other studies discovered that a highly expressed form of Rad51, known as a Rad51C paralog, was even more elevated in broad varieties of cancers. The viral delivery of diphtheria toxin A bound to a Rad51C promoter represented new insight on Rad51 but also introduced a new way of targeting overexpressed Rad51 in cancer cells. These early results demonstrated even greater cancer cell death compared to existing therapeutics that interfered with Rad51 or the experimental IBR-2 drug.

Given the evidence showing that Rad51 targeting could improve chemosensitivity and radiosensitivity, it was enlightening to review articles that reexamined existing drugs and demonstrated the role that these drugs had on improving conventional therapies through Rad51 targeting. The arching theme of these reviews suggested that Rad51 is a viable target, along with having value as diagnostic and prognostic factors, in wide varieties of cancers and are applicable at improving chemotherapy or radiotherapy both in-vitro and in-vivo. However, tests of cell survival, cell growth, chemosensitivity, radiosensitivity, tumor shrinkage, and side effects all have wide variance between studies and within studies, making it very difficult to make a unified conclusion. Of note is that very few studies tested for chemosensitivity even though chemotherapeutics also induce



DSB and Rad51 inhibition and impair DSB repair. It is clear from the data that the studies differ in design, which makes it difficult to compare drugs and cancer lines that are of the same category, which would require complex analysis and changes to studies to make an accurate conclusion. Future analysis may require additional inclusion of Rad51 testing and standardization of how Rad51 targeting is tested. Since the radiosensitizing mechanisms from Rad51 suppression in existing therapeutics were recently discovered and demonstrated in new studies, it is possible that the lack of testing for this protein may have confounded the results of previous studies. Additional studies and testing of Rad51 would benefit reviews of past and future studies of radiosensitizers and chemosensitizers, which may require accounting for Rad51 action on inhibiting DSB repair. Experimental therapeutics showed great results from targeting Rad51 function or using overexpression of Rad51 as a target for poisons, which increases the value of investigating techniques for targeting Rad51. Despite the varying data and differences in acquiring these data, there is general evidence that indicate use of a Rad51 inhibitor should be included with conventional therapies due overexpression of Rad51 levels in many different types of cancers, the influential roles of affecting growth rate along with permitting metastasis, and the ability for improvement in chemosensitivity and radiosensitivity without significant side effects.

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## CURRICULUM VITAE

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#### **Education**

Boston University School of Medicine, Boston MA  
M.A. **Medical Sciences**, Expected Graduation January 2015

Florida State University, Tallahassee FL  
B.S. **Biological Sciences**, April 2011

#### **Medically Relevant Experiences**

FriendshipWorks, Boston MA  
**Medical Escort Volunteer** (July 2014-Current)  
Provided round-trip assistance for elderly and disabled patients, aided with medication lists, documentation, registration, helped patients understand procedure instructions, served as liaison/advocate between patient and physician.

U.F. Health-Shands Hospital, Gainesville FL  
**Emergency Department Volunteer** (June 2013-December 2013)  
Provided customer service/assistance to patients and staff and maintained inventory.  
**O.R./Anesthesia Workroom Volunteer** (June 2013-August 2013)  
Aseptically prepared equipment, transported equipment, aided Anesthesia Technicians with O.R. preparation and turnover.

Devereux Florida-Statewide Inpatient Psychiatric Program, Tallahassee FL  
**Mental Health Technician** (July 2011-September 2011)  
Supervised and interacted with 8 adolescent patients, assisted with daily living activities, performed observation and documented patient status every 15 minutes.

Big Bend Hospice, Tallahassee FL  
**Facilities Volunteer** (August 2008 to July 2011)  
Provided companionship for patients and respite for caretakers.

## **Work Experiences**

Florida State University, Tallahassee, FL

**Lab Teaching Assistant** (August 2010-August 2012)

Instructed for Biology Lab, General Chemistry Lab, Organic Chemistry Lab, supervised 24 students per lab, held office hours, graded different assessments, kept record of student performance, and encouraged learning and lab safety.

Kerr and Downs Research, Tallahassee, FL

**Phone Interviewer** (May 2010-August 2010)

Collected data and recruited eligible participants for government surveys (~100 calls/hour).