CONTRADICTORY EFFECTS OF DASATINIB ON METASTASIS

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
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To my mom and dad, for teaching me the importance of education

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I would like to start at the beginning, with my mother and father. They deserve so much credit for whatever successful qualities I may have. They've taught me the importance of a good education, and how to budget time and money. They've taught me to be successful in school, in a career, and in life. Without their support, love, and guidance, I am sure I would not have gotten this far. I would like to thank my big brother for reminding me not to get so stressed out with the little details that I forget to enjoy life. My soon-to-be husband Chris Hughes has earned my interminable gratitude for unconditionally supporting, loving, and believing in me, regardless of my mental status on any given day.

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I also wish to thank those involved in the support and operations of this new master's program. The program has provided a unique learning opportunity to explore biotechnology and business, which surely will benefit me throughout my career. My fellow master's students have been an immense help, as well. As the inaugural class of the Translational Biotechnology program, we have relied on each other to navigate all the ins and outs of a new program.

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CONTRADICTORY EFFECTS OF DASATINIB ON METASTASIS

By

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Metastasis is the cause of 90% of deaths due to cancer, and significantly decreases quality of life in patients. Prevention of metastasis would greatly improve outcome, and there are currently no approved anti-metastatic therapies available.

Metastasis is a multistep, complex process, requiring survival in the bloodstream, invasion at a secondary site, proliferation, and induction of angiogenesis. Inhibition of any one of these steps may decrease metastases formation. Src is a non-receptor tyrosine kinase well-known to contribute to the metastatic potential of tumor cells. It does so through alteration of signaling pathways important to metastasis, including adhesion, migration, invasion, and angiogenesis. Elevated levels of Src are common in many cancer types, and have been correlated with tumor progression and poor patient prognosis. Because of its importance in metastatic phenotype, Src is a potential target in cancer therapy.

This thesis examines whether disruption of the Src signaling pathway could inhibit metastases formation. The Src inhibitor Dasatinib was evaluated *in vitro* and *in vivo* using the highly metastatic 4T1 murine mammary adenocarcinoma cell line.

Dasatinib is highly effective at inhibiting migration and invasion *in vitro*. Surprisingly, when combined with radiation therapy it increases radioresistance in the 4T1 cell line, an effect not seen in other cell lines. As determined with an intradermal model, angiogenesis was inhibited whether by *in vitro* treatment of the cells, or by *in vivo* treatment of the mice. By using a tail vein injection model to study metastasis, cells treated in culture were impaired in their ability to form metastases, but mice treated with Dasatinib developed significantly higher numbers of metastases, in a dose dependent manner.

In conclusion, in the mouse mammary 4T1 model Dasatinib was found to be not effective at inhibiting metastasis, and may actually facilitate the process. These results warrant further study due to the large number of cancer patients participating in Dasatinib clinical trials.

CHAPTER 1
INTRODUCTION

Cancer is the second leading cause of death in America. It is estimated that 40% of all Americans will be diagnosed with cancer at some point in their lives[1]. Despite the effort, research, and progress that has been made, cancer remains deadly.

Breast cancer is a particularly common type of cancer in America, with one in eight women receiving a diagnosis in her lifetime. When caught early, breast cancer is treatable. The five year survival rate for patients diagnosed with localized cancer is 98%. However, once the cancer has progressed or spread to distant locations throughout the body, treatment options become limited and the five year survival rate drops below 25%. Current therapeutic options for breast cancer include surgery, radiation therapy, chemotherapy, and targeted therapy[1].

The prevention of metastasis would have a significant impact on the survival of cancer patients. Currently, there are no approved therapies that address this need. This thesis examines the use of the Src inhibitor Dasatinib as an anti-metastatic agent in breast cancer.

Cancer

Cancer is one of the leading causes of death in developed countries. As we grow older, the likelihood of developing cancer increases. As our population ages, more and more people will be diagnosed with, and die from cancer.

A tumor can arise when a cell has acquired a certain set of mutations, including self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless reproductive potential, sustained angiogenesis, and tissue invasion

and metastasis. A cell that has these characteristics may develop into a tumor, and if left untreated, become fatal[2].

Microenvironment

As the tumor grows, it initially obtains its oxygen and nutrients by simple diffusion. An incipient tumor can grow to 2-3 cubic millimeters without inducing its own blood supply. At this size, regions of hypoxia or necrosis will have developed, and the tumor cannot grow further without inducing its own vasculature. Angiogenesis, or the development of new blood vessels, is a critical function in development, wound healing, and menstruation. Angiogenesis is also required for tumors to grow past a few millimeters in size. To do this, tumor cells secrete proangiogenic factors, the most potent being Vascular Endothelial Growth Factor (VEGF). Proangiogenic factors bind to receptors on nearby blood vessels, inducing vessel growth towards the tumor [Figure 1-1]. Once vascularization has occurred, the tumor can continue to grow[3].

Even after a tumor has developed its own blood supply, hypoxic regions are still present due to abnormalities in tumor vasculature. Due to constant stimulation with proangiogenic factors from the tumor, the vasculature cannot mature and normalize. While normal vasculature is evenly spread throughout tissue, tumor vasculature is disorganized, weak, leaky, and easily occluded. As such, there are areas of the tumor that are too far away from a vessel for sufficient oxygenation (chronic hypoxia), and areas that experience periods of time with no oxygen due to intermittent vessel perfusion (acute hypoxia)[4].

HIF-1α, or Hypoxia-Inducible Factor-1α, is a heterodimeric transcription factor that is stabilized in hypoxic conditions. It binds to certain promoter elements, leading to upregulation of a variety of genes, including those involved in angiogenesis, invasion,

proliferation, and metabolism. Due to the upregulation of those genes, the resulting tumor cell is more aggressive and metastatic[5]. In humans, hypoxia in primary tumors is correlated with a poorer prognosis[6, 7].

Metastasis

Metastasis is a process by which cells leave a primary tumor and form secondary tumors at distant locations [Figure 1-2]. Metastases account for about 90% of deaths due to cancer. In breast and prostate cancer patients, deaths from the disease are primarily due to metastasis. If breast cancer is detected prior to metastasis formation, it is very treatable. However, once tumor cells have spread to distant locations throughout the body, treatment options are limited[89].

Metastasis is a complex, multistep process. It begins with a cell leaving the primary tumor. Tumor vasculature is immature and leaky, and provides a facile route for a cell to leave the primary tumor and travel to another location in the body. A cell may leave the circulation in one of two ways. Most commonly, the cell becomes arrested within a capillary bed. Capillary vessels are designed for passage of red blood cells, so the much larger cancer cells often get trapped. Alternatively, the cell may adhere to the endothelial cells lining the interior of the blood vessel. Subsequently, the cell attempts to invade through the blood vessel and penetrate into the surrounding tissue. After a cell's successful exit from the vasculature, the newly formed micrometastasis may lay dormant for some time[9].

Once in its new location, the micrometastasis can grow to a few cubic millimeters in size without vascularization. Up to this size, the tumor obtains oxygen through simple diffusion, and is typically asymptomatic. As the tumor grows past 2-3 cubic millimeters in size, the innermost cells become hypoxic. In order to grow larger, the tumor must

induce angiogenesis to provide a blood supply. Secretion of VEGF and other growth factors stimulate the nearby vessels to sprout and vascularize the tumor. With a provision of oxygen and nutrients, the micrometastasis can now grow into an overt metastasis[3].

Anti-Angiogenic Agents

Anti-angiogenic agents are designed to inhibit the induction of angiogenesis by tumor cells. The rationale is that by inhibiting blood vessel development, the tumor is deprived of oxygen and nutrients, so cannot continue to grow in size. When combined with other anti-cancer agents, anti-angiogenic agents may be useful for slowing tumor growth[10]. As discussed later in this chapter, Src plays a role in regulating angiogenesis[11].

Src

The Src Family Kinases is a group of 9 non-receptor tyrosine kinases. The namesake of this group, Src, is the most famous and widely studied in the group. Src is inactive in most normal cells. However, when Src is active it plays a role in a number of cellular processes, including survival, proliferation, adhesion, angiogenesis, and most notably, motility and invasion [Figure 1-3][12].

Src protein and activity levels are elevated in a number of cancers, including breast cancer. Additionally, protein and activity levels appear to positively correlate with disease progression[12,13,14]. Because of the critical role that Src plays in metastasis-related signaling pathways, inhibition of its activity may offer a valuable therapeutic strategy.

Angiogenesis and Src

Angiogenesis is regulated in part by Src. While the main effects of Src activity are increased mobility and invasiveness, most Src inhibitors also show some anti-angiogenic activity as well. Src plays a role in the expression of VEGF, especially in hypoxia. Hypoxia increases Src activity and VEGF expression in fibroblasts, while c-Src$^{-/-}$ cells show decreased VEGF expression under the same conditions[15]. Additionally, the expression of VEGF correlates directly with the activity level of c-Src[16]. It is believed that when Src is activated by hypoxia, it causes HIF-1α and STAT3 to bind to the VEGF promoter element, resulting in increased VEGF production[11].

Therapeutics

Traditionally, breast cancer has been treated with a combination of surgery, chemotherapy, and radiation. Recently, targeted therapies have been combined with conventional therapy in order to specifically target tumor cells. Src inhibitors, as well as many other therapies, are being developed with the aim to improve survival and minimize adverse effects in cancer patients.

Dasatinib

One such targeted therapy is Dasatinib [Figure 1-4], an orally available, small molecule tyrosine kinase inhibitor that binds to and inhibits all the Src family kinase members. In addition, it potently binds to the BCR/ABL protein, as well as c-KIT, PDGFR, c-FMS and Ephrin A receptor [Table 1-1]. It was first developed as a BCR/ABL inhibitor, and it is approved by the FDA for the treatment of chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. Dasatinib is also currently in phase II clinical trials for the treatment of breast cancer, as well as prostate, colon, non-small cell lung cancer, melanoma, and many others[17].

4T1 Model

The 4T1 cell line is derived from a mammary adenocarcinoma tumor that spontaneously arose in a BALB/c mouse. Highly aggressive and invasive, it has many similarities to human metastatic breast cancer. After transplantation into the mammary fat pad of the mouse, tumor cells will spontaneously metastasize to the lymph nodes, lungs, liver, bone, and brain. Because of this, the 4T1 cell line makes a valuable model to study human metastatic breast cancer[18].

Research Outline and Rational

Metastasis is responsible for the majority of deaths due to cancer. In order for a tumor cell to metastasize, it needs to successfully complete a number of steps, including survival, invasion, angiogenesis, and growth. Inhibition of one or more of these steps may decrease the metastatic spread of tumor cells. Small molecule inhibitors, such as Dasatinib, allow for targeting of tumor cells without the cytotoxic side effects of traditional therapies.

The aim of this project was to inhibit metastases by interfering with the ability of the tumor cell to invade, a critical step in metastasis. With this in mind, the 4T1 model was chosen for its highly metastatic phenotype, and Dasatinib was used because of its potency as a Src inhibitor.

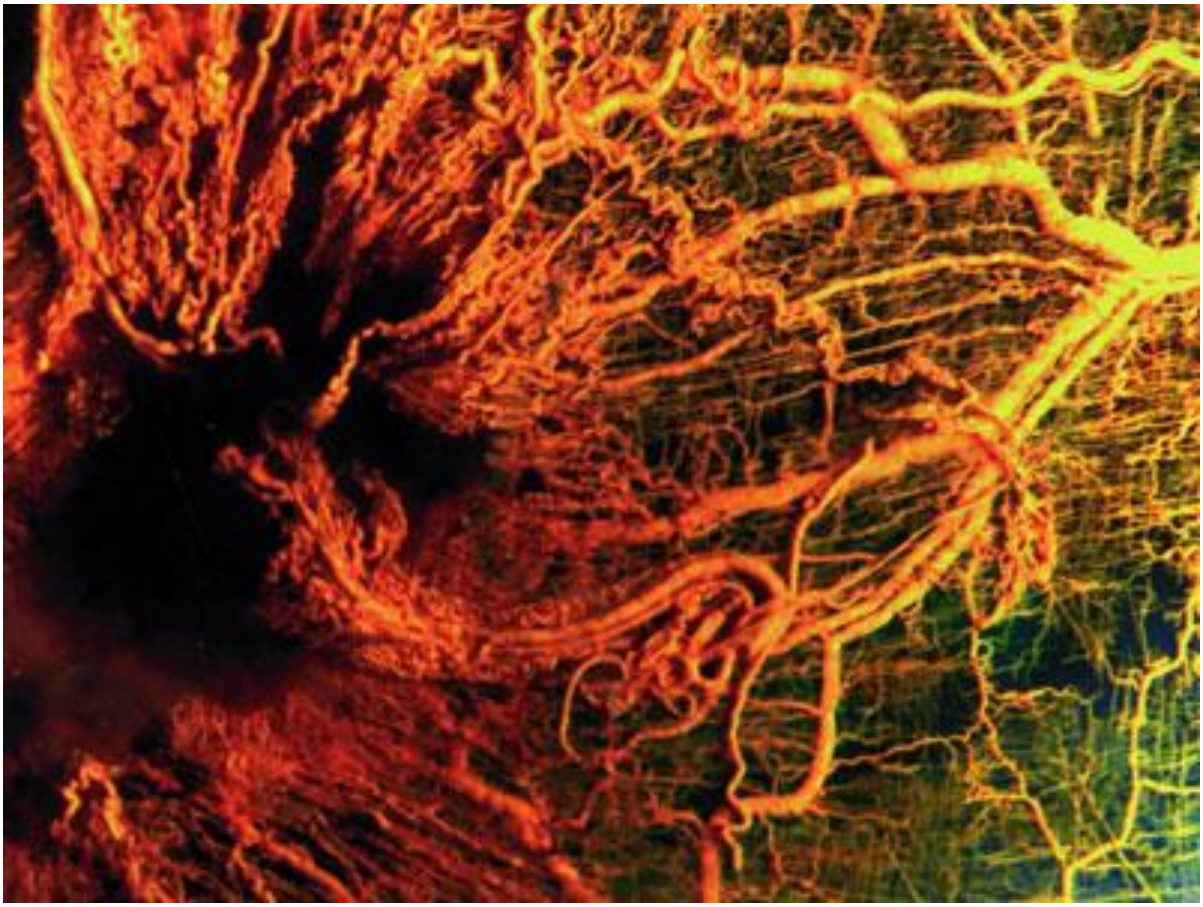

Figure 1-1. Micrograph of blood vessels growing towards a tumor[19]. Reprinted with permission.

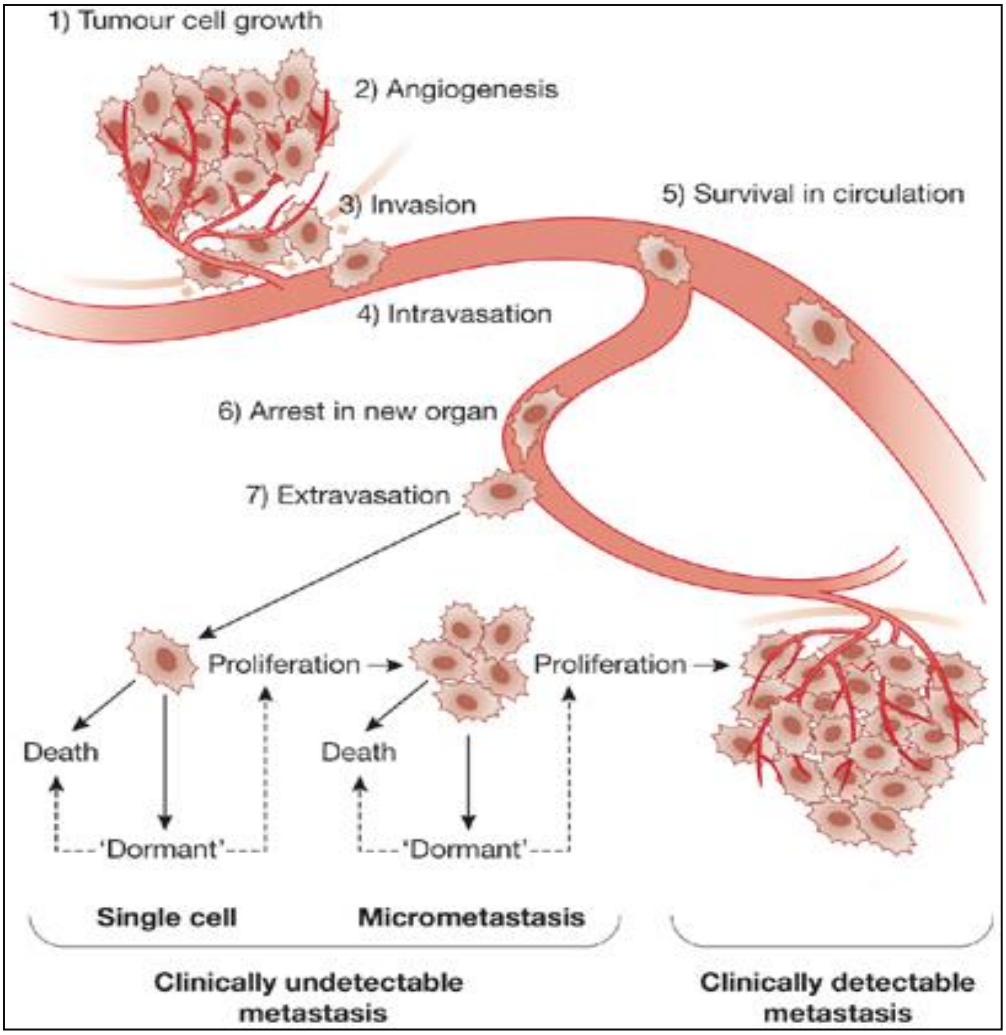

Figure 1-2. The metastatic cascade. Primary tumors shed cells into the circulation. Due to the harsh environment of the circulation, cells must extravasate within 24-48 hours. Once in the new secondary location, the cell with either die, lay dormant, or grow. After it has grown to 1-2 mm^3, the metastasis must induce angiogenesis to continue to grow in size[20]. Figure reprinted by permission from Macmillan Publishers Ltd: EMBO Reports. McGee et. al., EMBO Reports; 7(11): 1084–1088, copyright 2006.

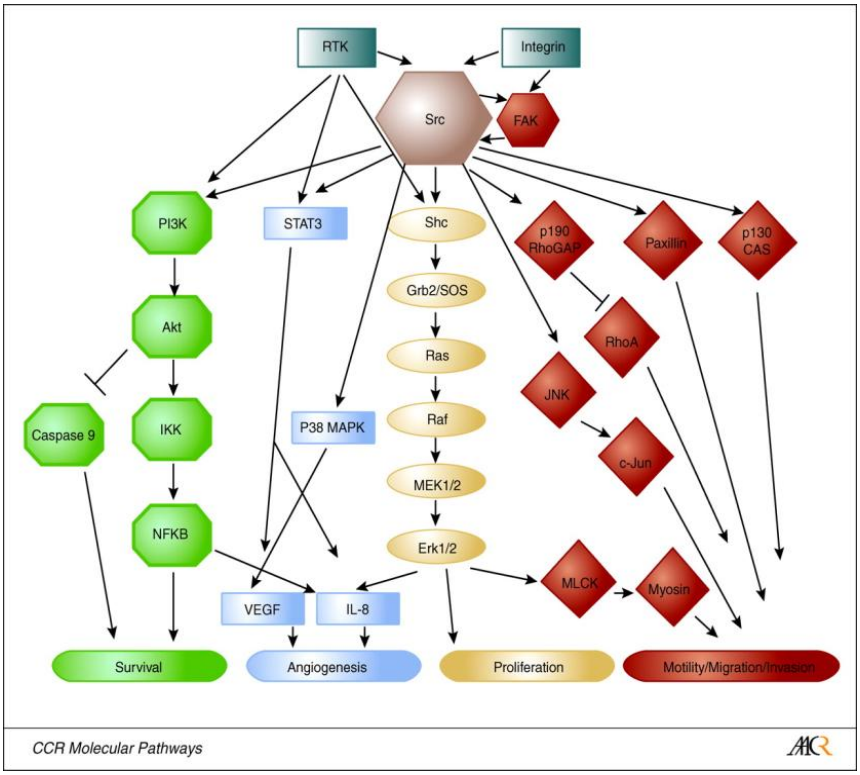

Figure 1-3. Src signaling pathways. Src is a non-tyrosine kinase receptor, and a member of the Src family kinases. It plays a major role in the migratory and invasiveness of tumor cells. It plays a lesser role in angiogenesis, and a minor role in survival and proliferation[21]. Reprinted with permission from the journal of Clinical Cancer Research. Copyright 2006, American Association for Cancer Research.

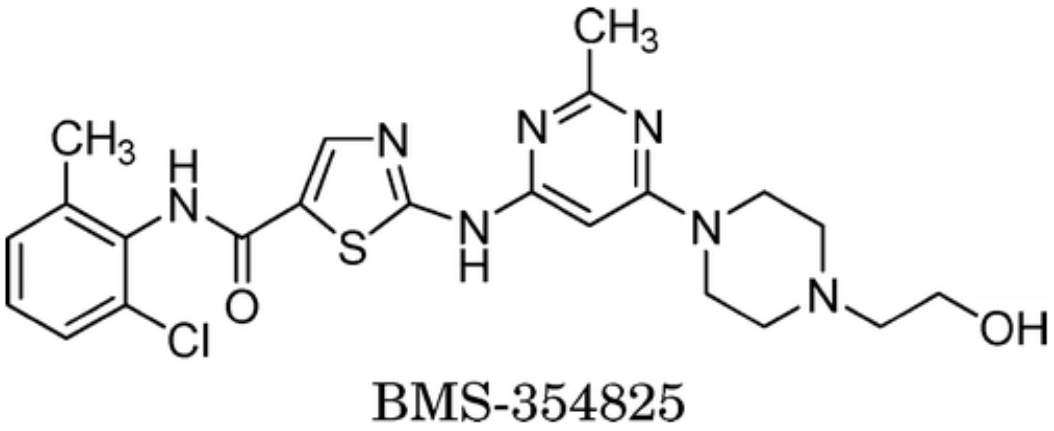

Figure 1-4. Chemical structure of Dasatinib (BMS-354825)[22]. Reprinted with permission from the Journal of Medicinal Chemistry. Copyright 2004, American Chemical Society.

Table 1-1. Binding affinity of Dasatinib to various targets[22-24].

Kinase	Enzyme IC50
BCR/ABL	<1.0 nM
Src	0.5 nM
Lck	0.4 nM
Yes	0.5 nM
c-KIT	5.0 nM
PDGFR	28 nM
c-FMS	4.1 nM
Eph A	17 nM

CHAPTER 2
IN VITRO ASSESSMENT OF DASATINIB ACTIVITY

Background

Src contributes to the metastatic phenotype of tumor cells by influencing migration, invasion, survival, and angiogenesis[12]. Src belongs to the Src family kinases, a group of 9 non-receptor tyrosine kinases. In most normal cells, Src is inactive. However, in many types of cancer, activity levels and protein levels of Src are elevated in comparison to normal tissues. Additionally, there is evidence that the activity level of Src correlates with the progression of the disease. Src is believed to contribute to the ability of cancer cells to metastasize.

Recently, interest in Src has spurred the development of several small molecule Src inhibitors. Pharmacological inhibition of Src may reduce the metastatic ability of tumor cells. Patient studies suggest that the incidence of metastasis may be inversely correlated with overall survival and quality of life. By inhibiting Src, it may be possible to reduce metastasis, and thereby improve overall host survival. To observe how Src inhibition affects metastasis, we used Dasatinib, a potent, orally available small molecule Src inhibitor[17]. Dasatinib has received FDA approval for its potent inhibition of the BCR/ABL fusion protein. It is currently used to treat chronic myelogenous leukemia and Philadelphia-positive acute lymphoblastic leukemia; both leukemias are driven by the BCR/ABL fusion protein.

The goal of the studies described in this chapter was to examine the effects of *in vitro* Dasatinib treatment on 4T1 tumor cells and normal endothelial cells.

Material and Methods

Cell Culture

4T1 murine mammary adenocarcinoma cells were cultured in Dulbecco's Modified Eagle's Media (DMEM), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin-streptomycin. Cell culture supplies were obtained from Invitrogen Corporation (Carlsbad, CA, USA). Cells were incubated at 37°C at 5% CO_2.

Human microvascular endothelial lung cells (HMVEC-L) were cultured in EBM-2-MV media from Lonza, and incubated at 37°C at 5% CO_2.

Drug Preparation

For *in vitro* use, Dasatinib (BMS 354825) was dissolved in DMSO at a concentration of 10 mM and stored at -20°C. Subsequent dilutions were made in PBS immediately prior to use.

For *in vivo* use, Dasatinib was dissolved in 80 mM citrate buffer and delivered by oral gavage at 0.01 mL/gram. A stock solution of 30 mg/mL was prepared weekly and stored at 4°C. Subsequent dilutions were made daily.

Cell Growth

4T1 cells ($7x10^3$) were seeded into 60 mm plates. Dasatinib was added (0.05 µM to 10 µM) 24 hours after plating the tumor cells. At various times thereafter, cells were counted with a hemocytometer. Three plates from each treatment group were counted at each time point.

Cell Survival

4T1 cells were treated with various doses of Dasatinib (0.05 µM to 10 µM) for 24 hours. Cells were harvested and plated at three different densities in 60 mm dishes.

Five days later, plates were stained with crystal violet dissolved in 200-proof ethanol. A light microscope was used to count colonies with $\geq$ 50 cells.

Migration

4T1 cells ($4x10^4$) plus drug were plated in 24 well modified Boyden chambers (BD Biosciences, San Jose, CA) in triplicate. When seeded in the chamber, cells will naturally migrate to the opposite side of the membrane. After 24 hours, any cells remaining inside the insert were removed using a cotton swab. Inserts were stained with crystal violet, and cells that successfully migrated were counted using a light microscope at 5X magnification [Figure 2-1]. The results of three independent experiments are shown.

Invasion

4T1 cells ($8x10^4$) plus drug were plated onto Matrigel-coated invasion chambers (BD Biosciences, San Jose, CA), in triplicate. Media inside the chamber was serum-free. Chambers were placed into wells of a 24 well plate containing media supplemented with 10% FBS as a chemoattractant. After 24 hours, cells remaining inside the inserts were removed using a cotton swab and stained with crystal violet. Cells that successfully invaded through the Matrigel were stained and counted using a light microscope at 5X magnification. The results shown in Figure 2-2 represent the average of three independent experiments.

Tube Formation

HMVEC-L cells at 60% confluency were treated with Dasatinib (0.05 µM to 1 µM) for 24 hours. Cells ($4x10^4$) were harvested and seeded onto Matrigel, and incubated for 24 hours to allow tube formation.

Western Immunoblotting

Western immunoblotting was used to assess the effect of Dasatinib on Src, p-Src, and downstream effecter proteins. After 24 hour Dasatinib treatment, cells were washed with cold PBS three times. Media and PBS rinses were centrifuged to recover non-adhered cells. Cells were harvested and lysed in RIPA buffer (50 mM HEPES, pH 7.4; 150 mM NaCl; 1% Triton X-100; 0.1% SDS; 0.5% sodium deoxycholate; 1 μM sodium orthovanadate; 5 μM EDTA; 5 μM sodium fluoride) containing a 1:20 dilution of mammalian protease inhibitor cocktail (Sigma-Aldrich. St. Louis, MO). Cell lysates were centrifuged at 14,000 rpm, 4°C for 10 minutes. A Bradford assay (BioRad Laboratories, Hurcules, CA) was used to assess total protein concentration. Lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to Trans-Blot® Transfer Medium nitrocellulose membrane (BioRad Laboratories, Hurcules, CA). Membranes were blocked in 5% nonfat dry milk in Tbs-t for 1 hour at room temperature on a shaker. Membranes were then incubated overnight at 4°C with primary antibodies for pSrc [Y423-mouse/Y416-chicken/Y419-human], total Src, pPaxillin [Y118], pAkt [Y308], pERK1/2 [T202/Y204] (Cell Signaling Technology, Inc., Danvers, MA), pFAK [Y861] (Invitrogen, Camarillo, CA). Membranes were incubated at room temperature for one hour with goat anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) to allow band visualization with chemiluminescence (ECL, GE Healthcare, Buckinghamshire, UK).

In Vivo Investigations

Intradermal angiogenesis assay

All animals were cared for according to the University of Florida Institutional Animal Care and Use Committee. Five to six week old female athymic *nu/nu* mice were obtained from Frederick Laboratories, and provided with food and water *ad libitum*. Mice were injected intradermally at four ventral sites with $1x10^5$ 4T1 cells that had been treated with Dasatinib for 24 hours. On the third day, mice were euthanized and skin flaps were removed. Vessels growing towards tumor nodules were counted under a light microscope.

Experimental metastasis assay

Five to six week old female BALB/c mice were obtained from Harlan Laboratories, and provided with food and water *ad libitum*. Mice were injected via tail vein with $1x10^5$ 4T1 cells that had been treated with 0, 50 nM, or 100 nM Dasatinib for 24 hours. On the 15^{th} day, mice were euthanized. Lungs were removed and placed in Bouin's solution (Sigma-Aldrich). Twenty-four hours later, the Bouin's solution was replaced with 70% isopropyl alcohol. Lung nodules were counted under a light microscope.

Results

The Effect of Dasatinib Treatment on Growth in the 4T1 Cell Line

Cytostatic agents, such as Dasatinib, are designed to affect the migration and invasion of tumor cells. Inhibition of growth indicates an undesired cytotoxic effect. In these studies the effect of Dasatinib treatment on 4T1 breast cancer cells was assessed. The data indicate that doses of Dasatinib below 1 μM did not have a significant effect on 4T1 cell growth [Fig. 2-3]. Furthermore a clonogenic cell survival

assay confirmed that doses of 500 nM and below were non-cytotoxic to these tumor cells [Fig. 2-4].

Dasatinib Treatment Inhibits Phosphorylation of Src and Downstream Proteins in the 4T1 Cell Line

Western blot analysis demonstrated that Dasatinib inhibited the phosphorylation of Src in a dose dependent manner, without effecting total levels of Src [Fig 2-5]. It also inhibited the phosphorylation of several downstream proteins, including Fak, Pax, Stat3, and Met in a dose dependent manner.

Migration and Invasion Are Inhibited by Dasatinib Treatment

To evaluate the functional effects of Dasatinib on tumor cells, migration and invasion assays were used. The ability of 4T1 cells to migrate and invade over 24 hours was significantly impaired by drug treatment [Fig 2-6]. At doses of 50 nM and 100 nM, Dasatinib inhibited migration by 56.8% and 90.6% respectively. At these doses Dasatinib inhibited invasion by 83.0% and 85.0% respectively.

Endothelial Tube Formation Is Not Affected by Dasatinib Treatment

In vitro data indicated that Dasatinib impaired the ability of 4T1 cells to migrate and invade, so a tube formation assay was used to explore the effects on normal endothelial cell function. The results showed that Dasatinib had no significant effect on tube formation at doses which are cytostatic to tumor cells [Fig. 2-7].

4T1 Cells Treated *In Vitro* Have a Reduced Ability to Induce Angiogenesis *In Vivo*

Cells were treated *in vitro* with 50 and 100 nM Dasatinib for 24 hours, and then injected intradermally into *nu/nu* mice. After 3 days, mice were euthanized and the skin flaps were removed. The results showed that cells that had been exposed to 100 nM Dasatinib had a significantly reduced ability to induce angiogenesis [Fig. 2-8].

4T1 Cells Treated *In Vitro* Have a Reduced Ability to Form Lung Metastases *In Vivo*

4T1 tumor cells were treated *in vitro* with 100 nM Dasatinib for 24 hours, and then injected into the tail veins of BALB/c mice. After 15 days, mice were euthanized, the lungs excised, and nodules on the lungs were counted. Tumor cells that had been exposed to Dasatinib formed ~5-fold fewer lung nodules than did untreated tumor cells [Fig. 2-9].

Discussion

Src is believed to contribute to the metastatic phenotype of cancer cells of many types of malignancies, by increasing the invasive, migratory, and angiogenic capability of tumor cells. Because of the importance of Src in the progression and invasion of tumor cells to the metastatic phenotype, several Src-targeting agents have been developed. The studies described in this chapter assessed the *in vitro* effects of the small molecule inhibitor Dasatinib on 4T1 cells. We determined that Dasatinib effectively inhibits phosphorylation of Src in a dose dependent manner, at sub-cytotoxic doses. From there, we performed various assays to examine if this molecular inhibition translated to a functional effect.

The ability to migrate and invade is critical for a tumor cell to successfully metastasize. After the cell leaves the primary tumor and enters the circulation, it must be able to invade out of the vasculature and into the surrounding tissues. Because elevated Src activity is known to increase migration and invasion, inhibiting Src may inhibit these functions and result in fewer metastases. In the *in vitro* migration and invasion assays, Dasatinib treatment resulted in decreased migration and invasion in a

dose dependent manner. The doses used did not affect normal endothelial tube formation.

Finally, we tested the effect of *in vitro* Dasatinib treatment on *in vivo* metastasis. 4T1 tumor cells exposed to 100 nM Dasatinib *in vitro* prior to injecting them into the tail veins of recipient mice were significantly impaired in their ability to form lung metastases.

This *in vitro* assessment demonstrates that Dasatinib effectively inhibits Src phosphorylation, as well as many functions important to cancer metastasis. This is consistent with published data. Based on our findings, we hypothesized that Dasatinib may have use as an anti-metastatic agent in an *in vivo* setting. This hypothesis was subsequently explored in Chapter 4 of this thesis.

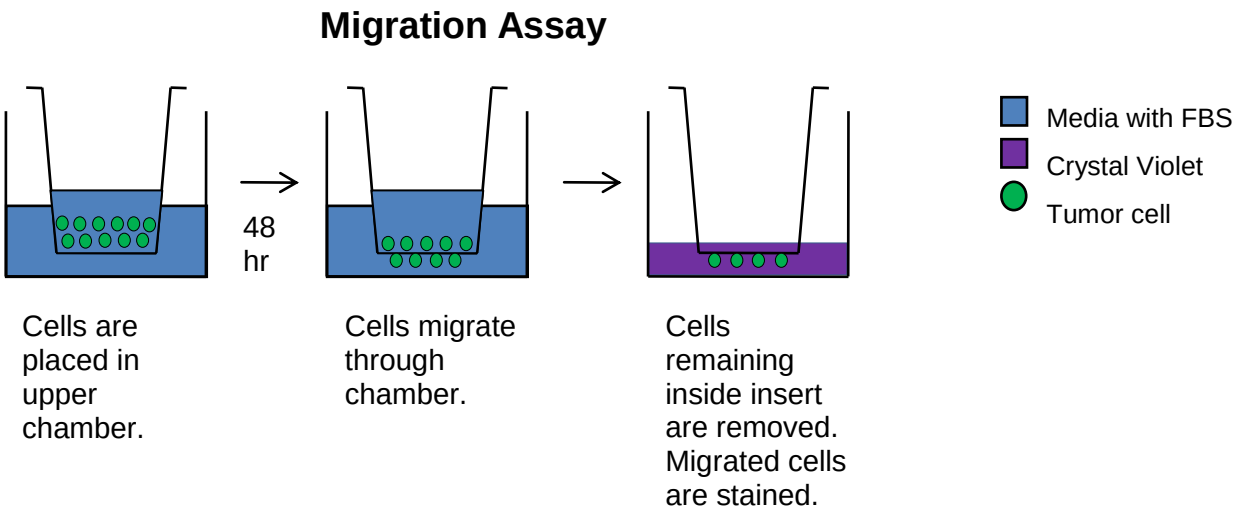

Figure 2-1. Schematic of migration assay.

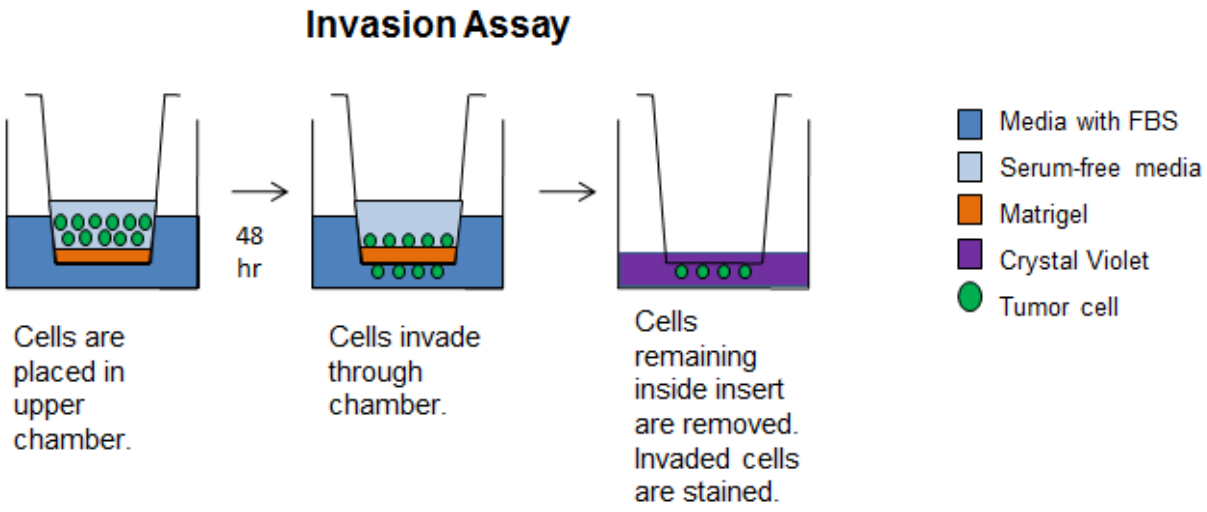

Figure 2-2. Schematic of invasion assay.

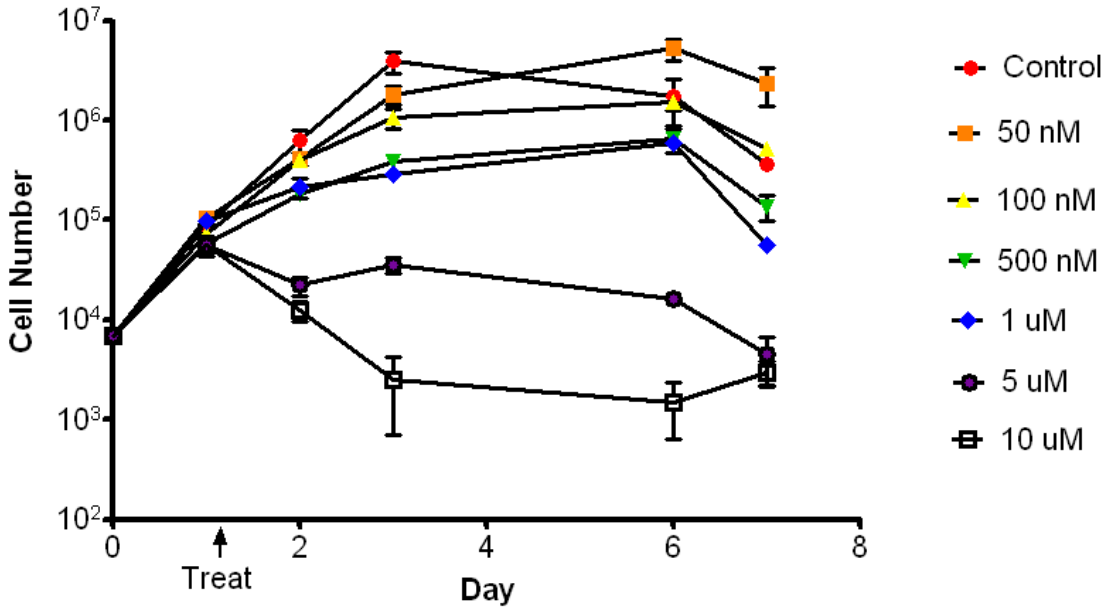

Figure 2-3. Effect of Dasatinib on 4T1 growth. Cells were treated with drug 24 hours after plating. At each time point, the number of cells from three dishes per drug dose were counted.

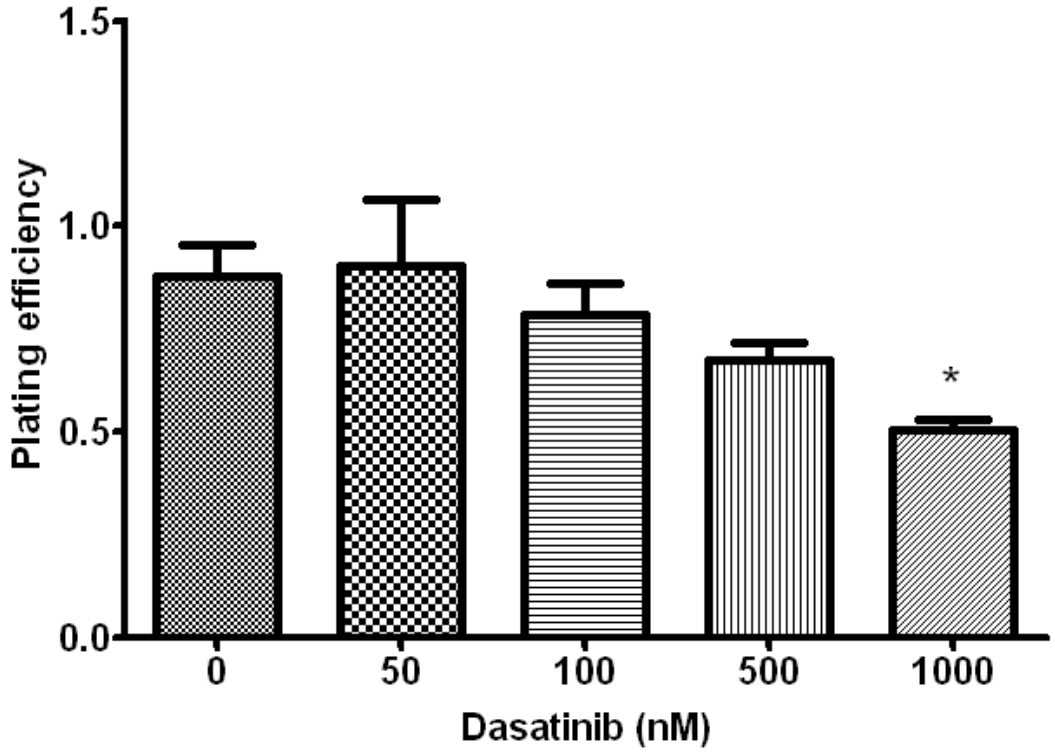

Figure 2-4. Clonogenic cell survival of 4T1 cells after 24 hour Dasatinib exposure. The data shown are the means and standard errors of three independent experiments. Analysis was done by two-tailed unpaired student's t-test. *$p<0.05$.

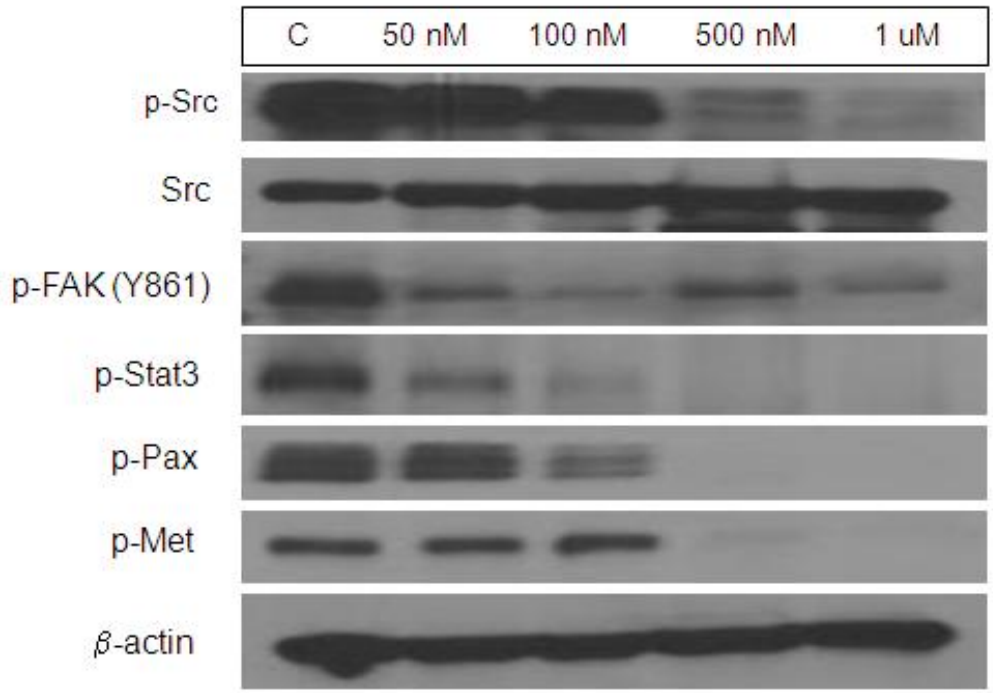

Figure 2-5. Effects of a 24 hour exposure of a range of Dasatinib doses on phosphoprotein expression of Src and down-stream effectors in 4T1 breast cancer cells.

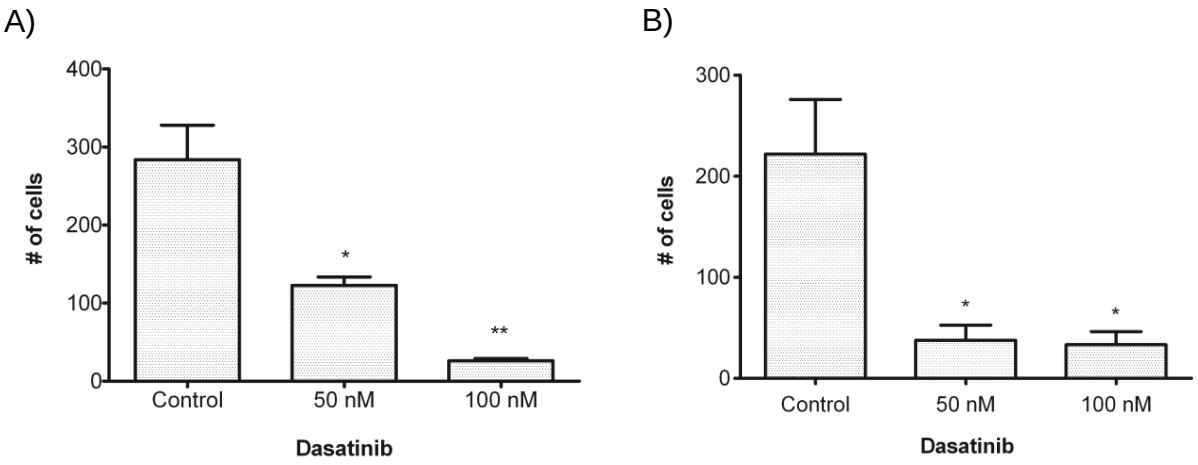

Figure 2-6. Effects of a 24 hour exposure of Dasatinib (50 nM or 100 nM) on migration and invasion of 4T1 breast cancer cells. A) Migration. B) Invasion. Two-tailed Student's t test was used for analyzing data in GraphPad Prism 5.0. *$p<0.05$, **$p<0.01$. The data shown are the means and standard errors of a combination of three independent experiments.

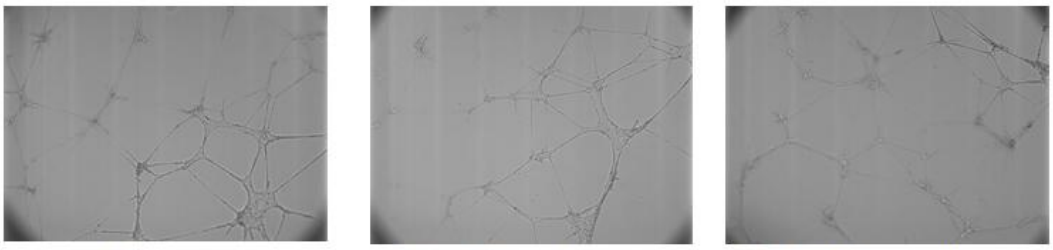

Figure 2-7. Effects of a 24 hour exposure of 50 nM and 100 nM Dasatinib on the ability of HMVEC-L cells to form tubes. Images were taken at 5x magnification.

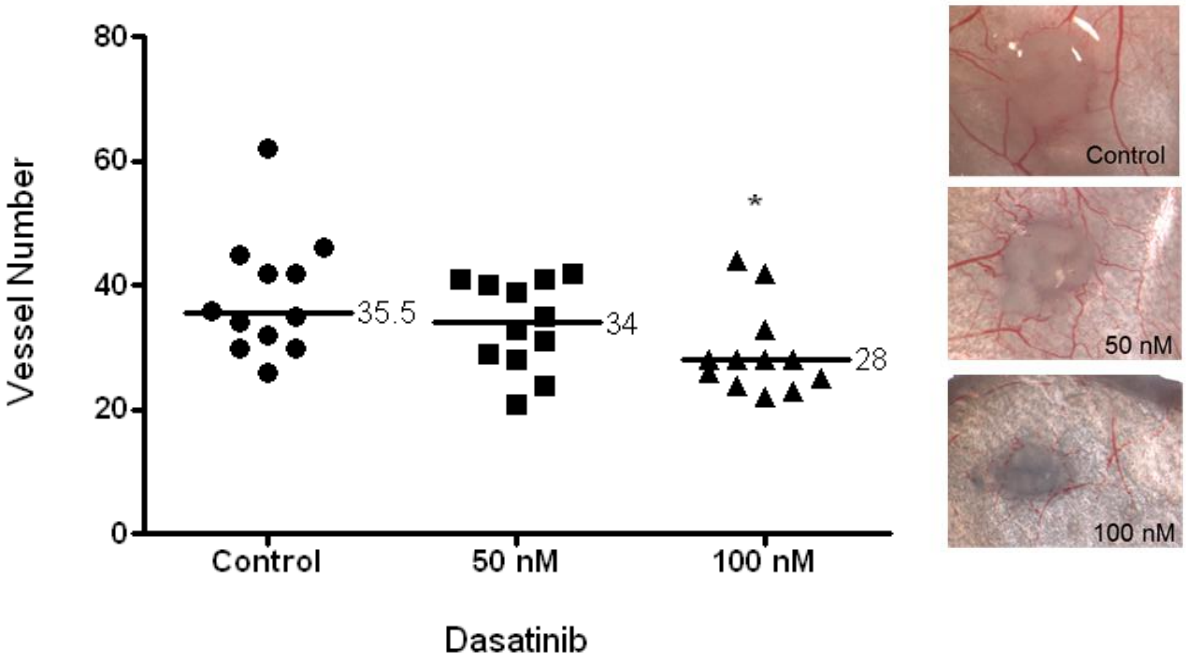

Figure 2-8. Effect of pretreating 4T1 tumor cells for 24 hours with Dasatinib (50 or 100 nM) on their ability to induce blood vessels as assessed using an intradermal angiogenesis assay 3 days after inoculating 10^5 4T1 tumor cells. Median values are shown. Wilcoxon matched pairs test was used to analyze data in GraphPad Prism 5.0. *****$p<0.05$.

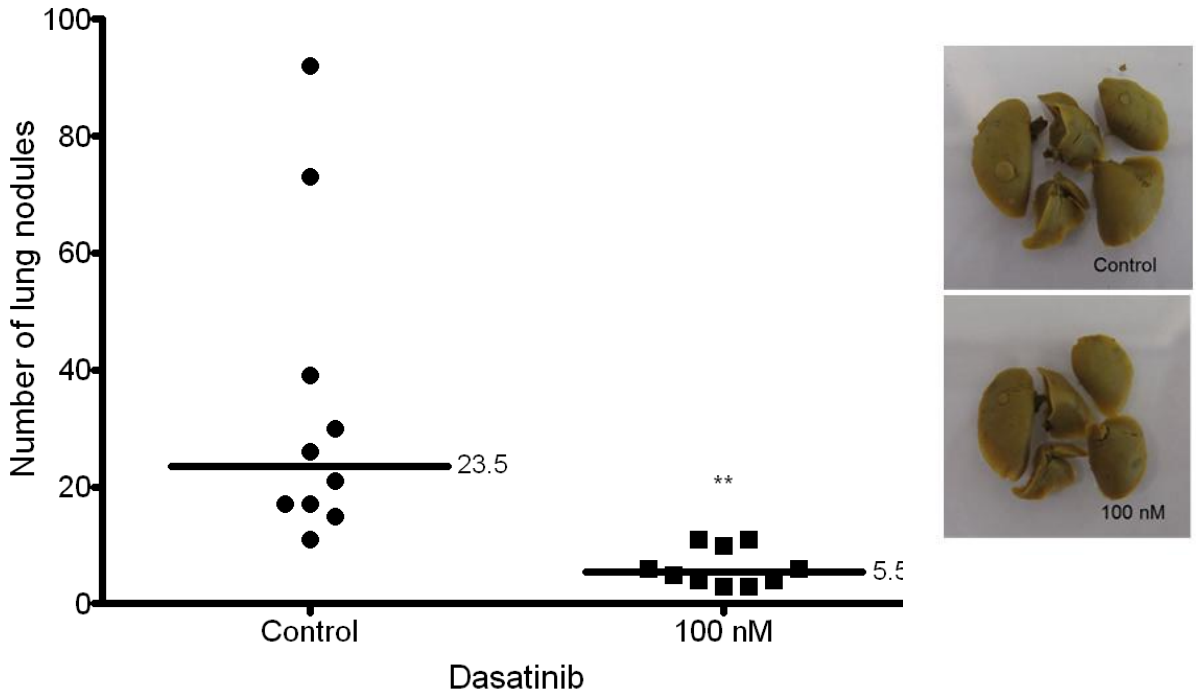

Figure 2-9. Effect of pretreating 4T1 breast cancer cells for 24 hours with 100 nM Dasatinib on their ability to form metastases in a BALB/c mouse model. This was assessed by using a tail vein injection assay with 10^5 4T1 cells. Median values are shown. Wilcoxon matched pairs test was used to analyze data in GraphPad Prism 5.0. **p<0.01.

CHAPTER 3
IN VITRO COMBINATION OF DASATINIB AND RADIATION

Background

When new cancer therapeutics are first introduced into the clinic, they are added to existing standard of care treatments. This means that they are typically combined with conventional therapy such as radiotherapy or chemotherapy in order to provide the clinical trial participants with the best foreseeable outcome. Conventional therapeutics for breast cancer include chemotherapy, surgery, and radiation.

Radiation is an effective mode of cancer therapy, and is commonly combined with surgery to kill cancer cells that may be remaining after removal of the primary tumor. Radiation therapy kills cells by causing damage, either direct or indirect, to the DNA molecules [Figure 3-1]. Direct damage occurs when a photon deposits its energy at the DNA molecule, removing an electron and thereby causing an ionizing event. This can result in single-stranded or double-stranded breaks. Indirect damage is more common, occurring when radiation ionizes water molecules to form free radicals. If created near the DNA, these highly reactive free radicals can attack the DNA and remove electrons. The cationic charge on the DNA molecule is very unstable, and may either be repaired through the transfer of a hydrogen by a thiol, or may bind to an oxygen molecule, which "fixes" the damage. Once oxygen is bound, replication machinery is unable to replicate the DNA normally. Cells that are unable to overcome DNA damage lose their clonogenicity- that is, their reproductive abilities[25].

While radiation therapy for breast cancer patients can be very effective, it typically is given in small daily doses and the entire treatment takes 6-8 weeks to complete. This is because the cells in a tumor are in various stages of the cell cycle. Cells in S-phase

are more radioresistant, while cells in M-phase are more radiosensitive. Radiation is given in frequent doses to allow the remaining viable tumor cells after treatment to progress through the cell cycle and out of the radioresistant phase. During the course of radiation therapy, the primary tumor is still present and continues to shed tumor cells into the circulation. These cells have the potential to develop into metastases. Use of an anti-metastatic agent during radiation therapy may inhibit the formation of new metastases, and thereby increase event-free survival in these patients. This chapter explores the *in vitro* combination of Dasatinib with radiation therapy in several tumor cell lines of varying origins, including breast cancer.

Materials and Methods

Cell Culture

4T1 (murine breast carcinoma), MDA-MB-231 (human breast carcinoma), SCC7 (murine head and neck squamous cell carcinoma), and KHT (murine fibrosarcoma) cell lines were cultured in Dulbecco's Modified Eagle's Media (DMEM), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin-streptomycin. UM-SCC-1 (human head and neck squamous cell carcinoma) cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin-streptomycin, and 1% hydrocortisone. A549 (human non-small cell lung cancer) cells were cultured in RPMI media. Cell culture supplies were obtained from Invitrogen Corporation (Carlsbad, CA, USA). Cells were incubated at 37°C in 5% CO_2:95% air.

Drug Preparation

Dasatinib (BMS 354825) was dissolved in DMSO at a concentration of 10 mM and stored at -20°C. Subsequent dilutions were made in PBS immediately prior to use.

Radiation Cell Survival

Cells were treated with Dasatinib for 24 hours prior to being irradiated with a cesium-137 irradiator. Immediately after irradiation, cells were harvested and plated at three different densities in 60 mm dishes. In Figure 3-4, cells were irradiated first, treated with drug for 24 hours, then harvested and plated. After some time (4T1=5 days, SCC7=7 days, A549 and UM-SCC-1=10 days, MDA-MB-231=14 days), plates were stained with crystal violet dissolved in 200-proof ethanol. Cell lines were stained at different times due to differences in growth rate. A light microscope was used to count colonies of at least 50 cells.

Results

Dasatinib Does Not Have an Effect on Radioresistance in Most Cell Lines

There are currently clinical trials assessing Dasatinib in combination with radiation therapy in non-small cell lung cancer and head and neck cancer. To determine if Dasatinib had any effect on radiosensitivity *in vitro*, a variety of cell lines were assessed. 4T1 (murine breast carcinoma), A549 (human non-small cell lung cancer), UM-SCC-1 (human head and neck carcinoma), SCC7 (murine head and neck carcinoma), MDA-MB-231 (human breast carcinoma), and KHT (murine fibrosarcoma) cell lines were assessed (data not shown for KHT). Cell lines were treated with 100 nM Dasatinib for 24 hours, and subsequently exposed to a range of radiation doses (0-9 Gray). Dasatinib did not have an effect on radiosensitivity in any of these cell lines, except for the 4T1 cell line [Figures 3-2 to 3-6].

Dasatinib Treatment Confers Radioresistance in 4T1 Cells

4T1 tumor cells pretreated with 100 nM of Dasatinib for 24 hours were more radioresistant than cells not exposed to the drug [Figure 3-6]. To determine if this was a

dose-dependent response, cells were treated with increasing doses of Dasatinib for 24 hours, followed by irradiation with 9 Gray. As seen in Figure 3-7, cell survival and drug dose were positively correlated. A dose of 100 nM resulted in a greater than 1 log increase in cell survival of 4T1 cells.

Dasatinib Does Not Interfere with DNA Repair Mechanisms in 4T1 Cells

In order to test if Dasatinib was having an effect on DNA repair, as opposed to the initial DNA damage, cells were exposed to radiation immediately followed by 24 hour exposure to Dasatinib. The treated group did not display increased resistance [Figure 3-8].

Discussion

When introducing a new cancer therapeutic into the clinic, it is initially included in the currently used standard of care therapy. Radiation is commonly used in breast cancer treatment, and was a logical choice for combination with Dasatinib.

In order to observe the effects of Dasatinib on radiation therapy, we performed a clonogenic cell survival assay on several cell lines. We selected NSCLC and head and neck cancer lines because of clinical trials involving those tumor types. We also tested a human breast line so as to encompass both human and mouse breast lines in our study. Cells were pretreated with drug prior to irradiation in order to observe effects on DNA damage and repair. Our results showed that Dasatinib pretreatment did not affect the radiation response of any of the cell lines studied, with the exception of the 4T1 breast cancer cell line. In that cell line, pretreatment with the Src inhibitor resulted in radioprotection [Figure 3-6]. As seen in Figure 3-7, this effect was found to be dose dependent. In order to determine whether the enhanced cell survival was due to a radioprotective effect or a consequence of an altered repair process, 4T1 tumor cells

were irradiated, then exposed to 100 nM Dasatinib for 24 hours. In this experiment, Dasatinib did not affect the radiosensitivity of the cells, indicating that Dasatinib is not affecting the DNA repair mechanisms. These results lead us to believe that Dasatinib is having an effect on the initial damage to the DNA.

To gain a better understanding of the mechanisms underlying the Dasatinib-induced radiation resistance in the 4T1 line, additional experiments will need to be undertaken. It appears that its effect is occurring during the damaging of the DNA molecules. Certain molecules such as thiols or superoxide dismutase are present in cells to protect the DNA from damage. One possibility is that Dasatinib increases thiol or superoxide dismutase levels, thus protecting the cell from DNA damage. Dasatinib inhibits a range of targets, and it is possible that one of these targets is responsible for the increase in radioresistance. While c-KIT, PDGFR, c-FMS, and Src all protect cells from apoptosis, the Ephrin A receptor enhances apoptosis. Future experiments may include testing other agents that specifically affect BCR/ABL, Src, or other members of the Src family, in order to gain further insights into whether the radioresistant effect is particular to Dasatinib.

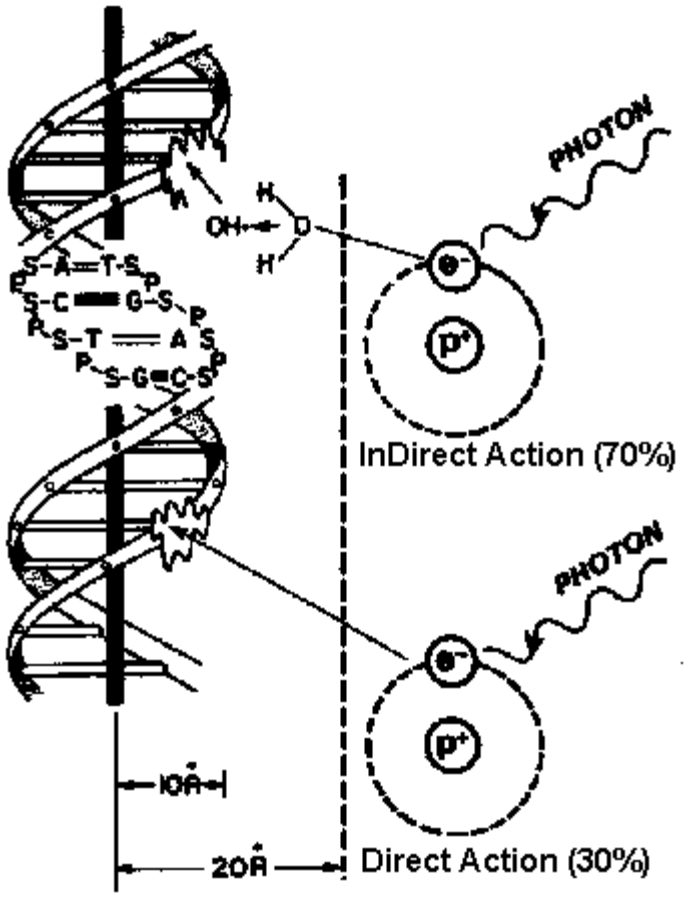

Figure 3-1. Schematic of indirect and direct radiation damage[26].

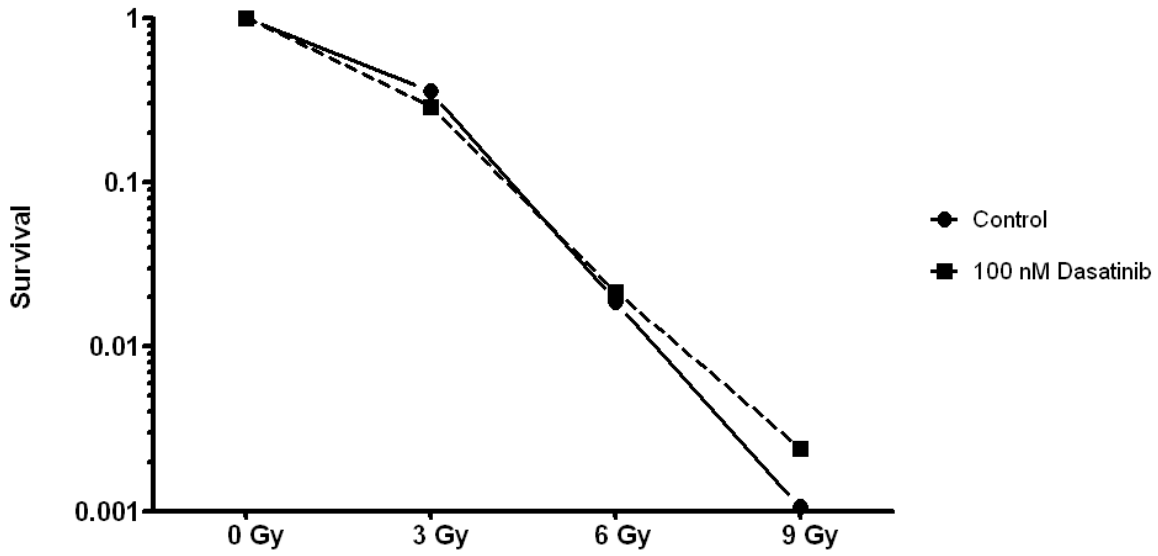

Figure 3-2. Effect of radiation on A549 cells treated with 100 nM Dasatinib. After 24 hour drug treatment, cells were exposed to 0, 3, 6, or 9 Gray radiation. Cells were then lifted and replated at 3 different densities in 60 mm dishes. After 10 days, the dishes were stained with crystal violet, and colonies of $\geq$50 cells were counted under a light microscope. The surviving fraction is shown in the graph.

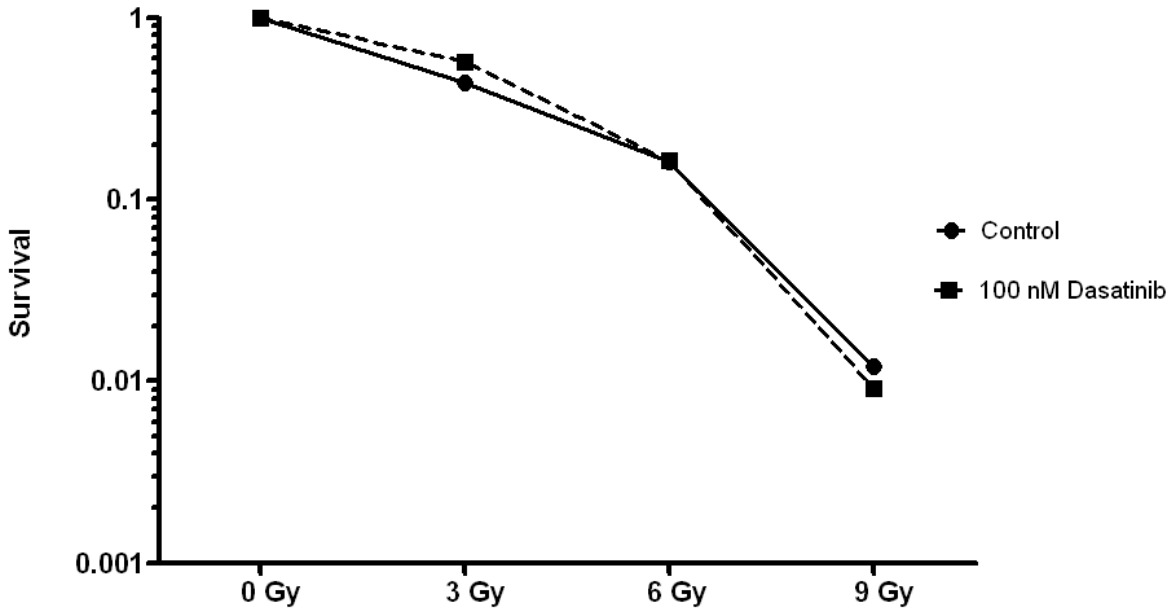

Figure 3-3. Effect of radiation on UM-SCC-1 cells treated with 100 nM Dasatinib. After 24 hour drug treatment, cells were exposed to 0, 3, 6, or 9 Gray radiation. Cells were then lifted and replated at 3 different densities in 60 mm dishes. After 10 days, the dishes were stained with crystal violet, and colonies of $\geq$50 cells were counted under a light microscope. The surviving fraction is shown in the graph.

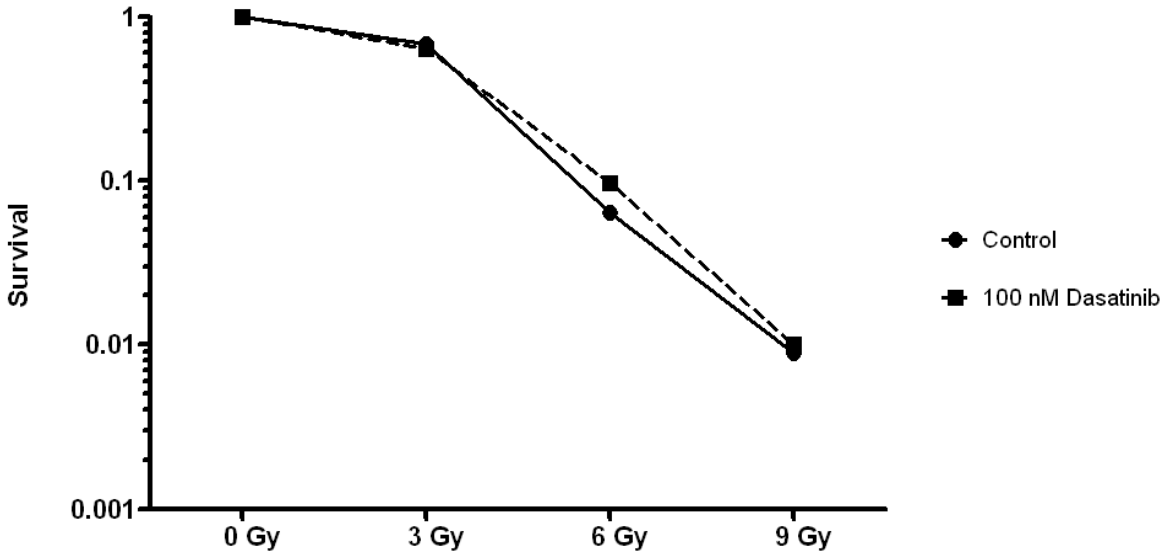

Figure 3-4. Effect of radiation on SCC7 cells treated with 100 nM Dasatinib. After 24 hour drug treatment, cells were exposed to 0, 3, 6, or 9 Gray radiation. Cells were then lifted and replated at 3 different densities in 60 mm dishes. After 7 days, the dishes were stained with crystal violet, and colonies of $\geq$50 cells were counted under a light microscope. The surviving fraction is shown in the graph.

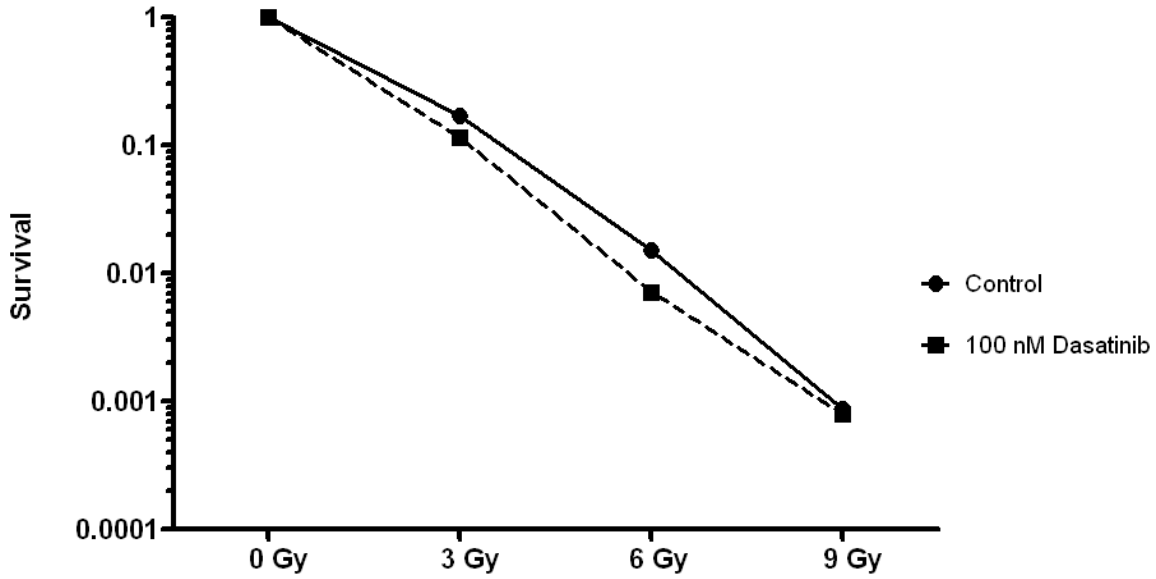

Figure 3-5. Effect of radiation on MDA-MB-231 cells treated with 100 nM Dasatinib. After 24 hour drug treatment, cells were exposed to 0, 3, 6, or 9 Gray radiation. Cells were then lifted and replated at 3 different densities in 60 mm dishes. After 14 days, the dishes were stained with crystal violet, and colonies of $\geq$50 cells were counted under a light microscope. The surviving fraction is shown in the graph.

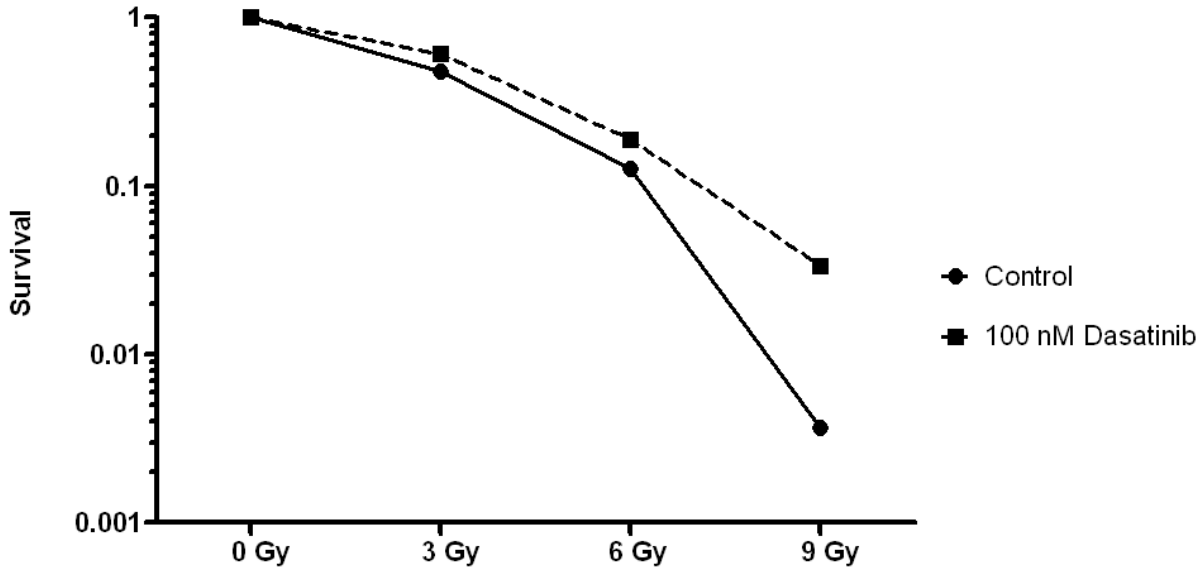

Figure 3-6. Effect of radiation on 4T1 cells treated for 24 hours with 100 nM Dasatinib. After 24 hour drug treatment, cells were exposed to 0, 3, 6, or 9 Gray radiation. Cells were then lifted and replated at 3 different densities in 60 mm dishes. After 5 days, the dishes were stained with crystal violet, and colonies of ≥50 cells were counted under a light microscope. In the graph, the surviving fraction is shown.

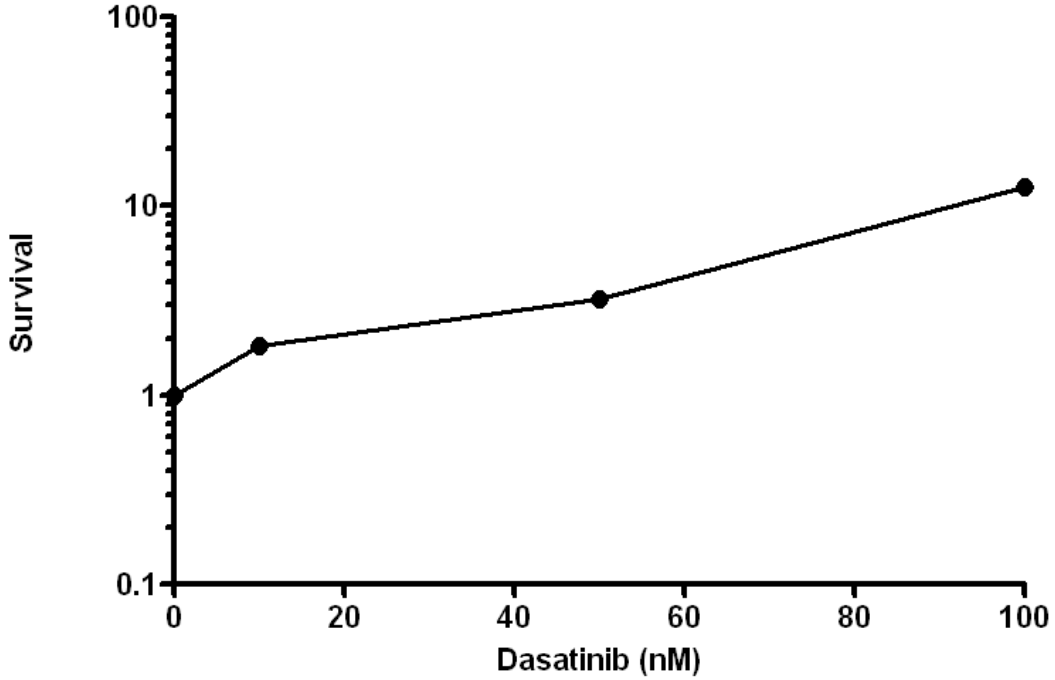

Figure 3-7. Effect of 9 Gray radiation on 4T1 cells treated with a range of Dasatinib doses (0-100 nM). After 24 hour drug treatment, cells were exposed to 0, 3, 6, or 9 Gray radiation. Cells were then lifted and replated at 3 different densities in 60 mm dishes. After 5 days, the dishes were stained with crystal violet, and colonies of $\geq$50 cells were counted under a light microscope. The surviving fraction is shown in the graph.

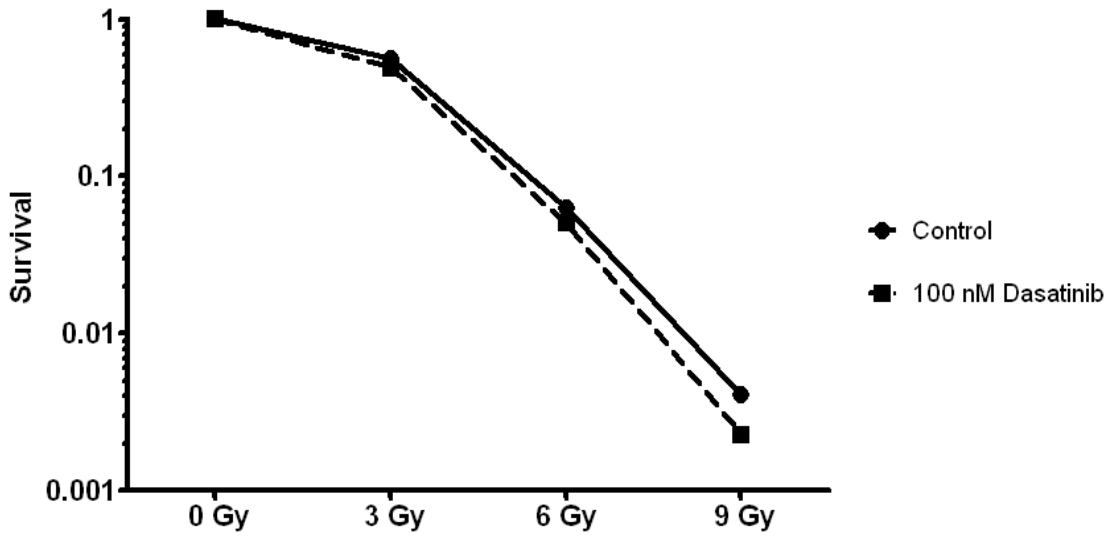

Figure 3-8. 4T1 cells treated with 100 nM Dasatinib immediately after irradiation for 24 hours. Cells were exposed to 0, 3, 6, or 9 Gray radiation, then immediately treated with 100 nM Dasatinib for 24 hours. Cells were then lifted and replated at 3 different densities in 60 mm dishes. After 5 days, the dishes were stained with crystal violet, and colonies of $\geq$50 cells were counted under a light microscope. The surviving fraction is shown in the graph.

CHAPTER 4
IN VIVO ASSESSMENT OF DASATINIB ACTIVITY

Background

The results of experiments described in the prior chapters of this thesis have demonstrated that Dasatinib potently inhibits cellular functions associated with metastatic activity in 4T1 cells *in vitro*. While this often is an indication of a drug's activity in animals and humans, the question still remains- does it affect the metastatic potential of tumor cells *in vivo*?

One function of tumor cells closely associated with their ability to successfully develop as secondary tumors at distant sites is the ability to induce angiogenesis [Figure 4-1]. To explore whether Dasatinib treatment could interfere with the initiation of new blood vessels by tumor cells *in vivo*, an intradermal assay was used. This assay determines the ability of tumor cells to induce angiogenesis. As mentioned in earlier chapters, Src inhibitors often have anti-angiogenic utility. We therefore were interested to determine if in the 4T1 breast cancer model, Dasatinib also had anti-angiogenic activity.

To test whether Dasatinib treatment could impair the dissemination of blood borne tumor cells, 4T1 breast cancer cells were directly injected into the blood stream of BALB/c mice [Figure 4-2]. This assay is a truncated version of the actual metastatic process. In the body, the tumor cell must leave the primary tumor, enter the circulation, extravasate, and initiate growth and angiogenesis. In this tail vein injection assay, the cells are being introduced into the blood stream, bypassing the first two steps. However, there are several advantages to using this assay to investigate the metastatic process. These include that the number of injected cells is known, and groups can be more

easily compared because variability is reduced. Thus this assay can be used to quickly and reproducibly determine the effects of the experimental agents on the ability for tumor cells to form lung metastases.

While Dasatinib is currently not used as an anti-metastatic agent, it has been used for years in the treatment of leukemia. Dasatinib is a potent BCR/ABL inhibitor, and was approved in June 2006 for the treatment of CML and Ph+ALL. It is highly effective at controlling BCR/ABL driven leukemias. Dasatinib has been or currently is in phase II trials for over a dozen solid tumor types, and the studies typically observe tumor growth in advanced patients. Given its effect on metastasis-associated cell functions, we hypothesized that Dasatinib may have *in vivo* activity.

Materials and Methods

Drug Preparation

Dasatinib was dissolved in 80 mM citrate buffer and delivered by oral gavage at 0.01 mL/gram. A stock solution of 30 mg/mL was prepared weekly and stored at 4°C. Dilutions were made daily.

Intradermal Angiogenesis Assay

Five to six week old female athymic *nu/nu* mice were obtained from Frederick Laboratories, and provided with food and water *ad libitum*. Mice were injected intradermally at four ventral sites with $1x10^5$ 4T1 cells, and orally gavaged with drug or vehicle at time of injection and once daily for two days thereafter. On the third day after cell inoculation, mice were euthanized and skin flaps were removed. Vessels growing towards tumor nodules were counted under a light microscope.

Experimental Metastasis Assay

4T1 model

Five to six week old female BALB/c mice were obtained from Harlan Laboratories, and provided with food and water *ad libitum*. Mice were injected via tail vein with $1x10^5$ 4T1 cells and treated orally with Dasatinib for five days following tail vein injection. Previous experiments with Dasatinib in this model in our laboratory showed that results were the same whether we treated for the first five days or the whole duration of the experiment. On the day 15, mice were euthanized. Lungs were removed and placed in Bouin's solution (Sigma-Aldrich). Twenty four hours later, the Bouin's solution was replaced with 70% isopropyl alcohol. Nodules on lungs were counted under a light microscope.

C3H model

Five to six week old female C3H mice were obtained from Frederick Laboratories, and provided with food and water *ad libitum*. Mice were injected via tail vein with $2x10^3$ KHT cells, and treated orally with Dasatinib for five days following tail vein injection. On day 17, mice were euthanized. Lungs were removed and placed in Bouin's solution (Sigma-Aldrich). Twenty four hours later, Bouin's solution was replaced with 70% isopropyl alcohol. Nodules on lungs were counted under a light microscope.

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0, using the Wilcoxon matched pairs test. A p value of less than 0.05 was considered statistically significant ($*p<0.05$).

Results

Dasatinib Inhibits Tumor Cell-Induced Angiogenesis *In Vivo*.

In order to determine the effect of Dasatinib on tumor cell-induced angiogenesis, an intradermal assay was utilized. Mice were injected intradermally with 10^5 4T1 cells that had been exposed to Dasatinib for 24 hours. Four days later, the mice were euthanized and the skin flaps were examined under a light microscope. Angiogenesis was inhibited in dose dependent manner when mice were treated with Dasatinib. The highest dose used, 15mg/kg, reduced the number of blood vessels by 40% [Figure 4-3].

Dasatinib Increases the Number of Lung Metastases in the 4T1-BALB/c Model.

BALB/c mice were injected with 4T1 cells and after 15 days the nodules on the lungs were counted. Figure 4-4 shows that treatment with increasing doses of Dasatinib lead to increasing numbers of lung metastases. For example, the 15 mg/kg dose of Dasatinib resulted in a ~10-fold increase in metastases, while the 30 mg/kg dose schedule resulted in a greater than 15-fold increase in metastases. This experiment was subsequently repeated using a higher number of injected tumor cells. Consistent with the initial experiment, the Dasatinib-treated group was found to have significantly more metastases than the control group [Figure 4-5]. However, no change in the size of the lung nodules was noted.

Dasatinib Has No Effect on the Formation of Lung Metastases in a KHT-C3H Model.

To determine whether the increased metastatic frequency observed in the 4T1 tumor model might be a cell line-specific phenomenon, a similar study was undertaken in the KHT mouse fibrosarcoma cell line in the C3H mice. The results showed that

unlike the observations made in the 4T1 model, Dasatinib did not affect the number of lung metastases in the KHT-C3H model [Figure 4-6].

Dasatinib Has an Effect on the Host, Creating a More Favorable Environment for Metastases.

To test if Dasatinib was affecting the host in a way that facilitated metastases, BALB/c mice were treated for 5 days with Dasatinib. Two mice from each group were euthanized at the end of treatment, and the lungs were stained with H&E. Twenty-four hours after the last drug treatment, the remaining mice were injected with 4T1 tumor cells and the number of lung metastases subsequently formed was quantified. The results showed that mice that had been pretreated with Dasatinib developed significantly higher numbers of lung metastases than did the mice in the untreated control group. Once again, there did not appear to be any differences in the size of the lung metastases. However, the histology (H&E staining) of the lungs failed to demonstrate any obvious differences between the lung tissues of control and Dasatinib treated mice.

Discussion

Contrary to the *in vitro* data shown in Chapter 2, *in vivo* treatment with Dasatinib appears to have no beneficial impact on the formation of lung metastases. While it did interfere with angiogenesis, Dasatinib also dramatically increased the frequency of metastases following tail vein injection of 4T1 breast cancer cells into the BALB/c mouse model. This effect was clearly dose dependent in this tumor model. To examine the generality of this observation, similar experiments were carried out in the KHT fibrosarcoma cell line injected into C3H mice. The results from that experiment showed

there was no significant difference between the numbers of lung colonies formed in Dasatinib treated and control groups.

We hypothesized that the drug may be affecting the host in such a way that the tumor cell can more readily establish secondary growth in the lungs. To study this, mice were treated with Dasatinib for five days. Twenty-four hours after the last dose, mice were injected via tail vein with 4T1 cells. Dasatinib has a short half-life *in vivo*; after 24 hours the drug is cleared from the mouse's system[27]. As seen in Figure 4-7, the number of lung metastases was increased over 2-fold in mice that had been pretreated with Dasatinib prior to tumor cell injection. There did not appear to be a difference in the size of the lung metastases, indicating the drug is not having an effect on tumor growth. This result supports the notion that Dasatinib is having an effect on the host that facilitates metastases formation. How this occurs is unknown. H&E staining was performed on lungs after mice had been treated with Dasatinib, but these failed to indicate obvious damage. However, it is possible that there are changes in the lung tissue too subtle to be detected by H&E staining. Future experiments could include EM staining to observe less obvious damage. Another possible test could include a bronchoalveolar lavage to quantify the levels of macrophages, lymphocytes, and neutrophils in the lungs.

Another possibility is that Dasatinib is not acting on the lungs, but on some other part of the mouse's body. One possibility is that the drug is elevating cytokines such as PlGF, TGF-α, EGF, or FGF that increase the metastatic potential of the tumor cells. Future experiments could include measuring blood levels of cytokines after Dasatinib treatment in this mouse strain. One experiment currently planned involves injecting nude mice with 4T1 cells via tail vein and treating the mice with Dasatinib. Another

possible experiment includes testing the EMT6 cell line (a BALB/c derived cell line) in the Dasatinib-treated BALB/c mouse. These experiments would assist us in further understanding the effects of the 4T1 cell line and BALB/c host in the previous experiments.

In conclusion, Dasatinib does not inhibit metastasis formation *in vivo*. More worrisome, in the 4T1 mammary carcinoma model treatment with this agent actually increases the number of lung colonies formed. It is estimated that about 33,000 patients have taken Dasatinib; this figure does not include the many patients participating in clinical trials. Given the current interest in Dasatinib as an anti-cancer therapy, these disturbing results warrant further investigation.

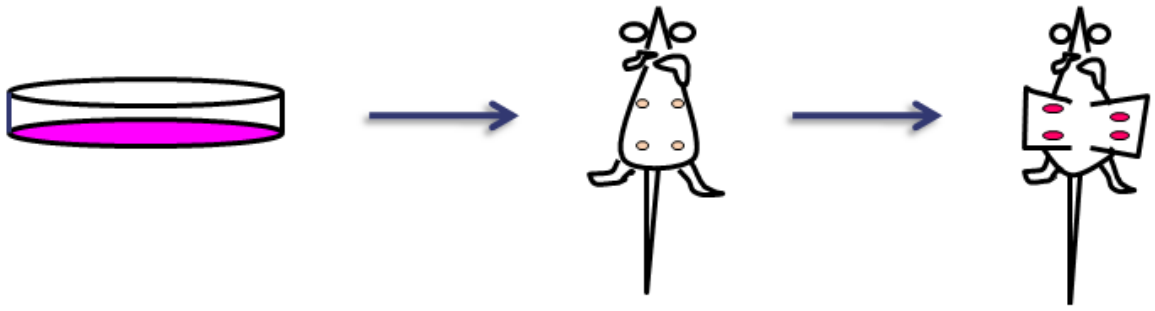

Figure 4-1. Schematic of intradermal assay. Cells were injected intradermally at four ventral sites on the mouse. Mice were treated with vehicle or drug on the day of injection, and for the following two days. On the third day after injection, the mice were euthanized, skin flaps removed, and the number of vessels intersecting each nodule were counted under a light microscope.

Figure 4-2. Schematic of lung metastasis assay. Cells were injected via tail vein. The vasculature carries the cells to the lungs, where most are arrested. After two weeks, the mice were euthanized, the lungs removed, and the number of nodules on the lungs were counted under a light microscope.

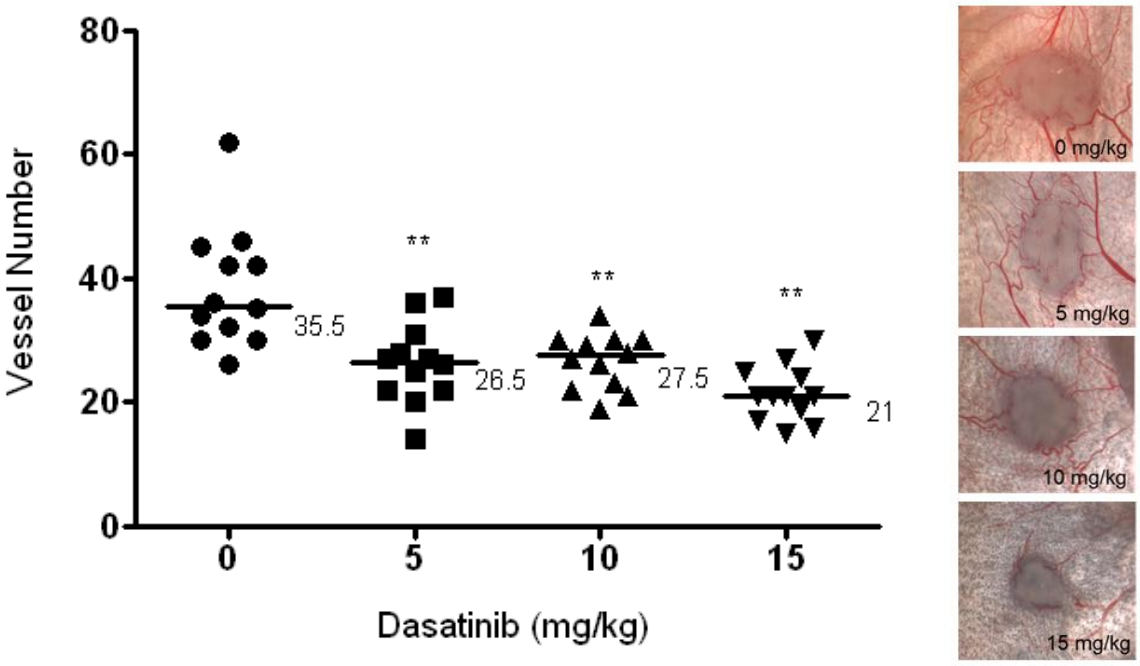

Figure 4-3. Intradermal assay: BALB/c mice. Mice were injected intradermally with 10^5 4T1 cells, and treated subsequently for three days with a range of Dasatinib doses. Each point on the graph represents the number of vessels intersecting a single nodule. Wilcoxon matched pairs test was used to analyze data in GraphPad Prism 5.0. ****p<0.01. Median values are shown.

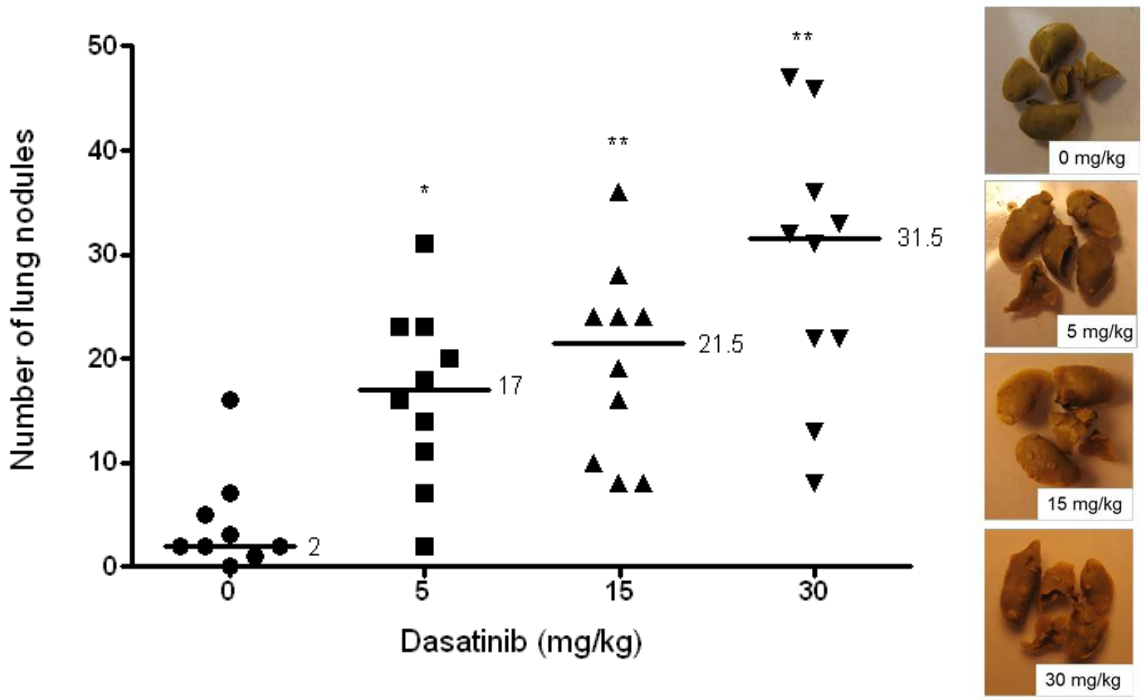

Figure 4-4. Lung metastasis assay: BALB/c mice injected with $5x10^4$ 4T1 cells. Mice were injected with $5x10^4$ 4T1 cells, and treated subsequently for five days with a range of Dasatinib doses. Each point on the graph represents the number of nodules found on a single set of lungs. Wilcoxon matched pairs test was used to analyze data in GraphPad Prism 5.0. *****$p<0.05$, **$p<0.01$. Median values are indicated.

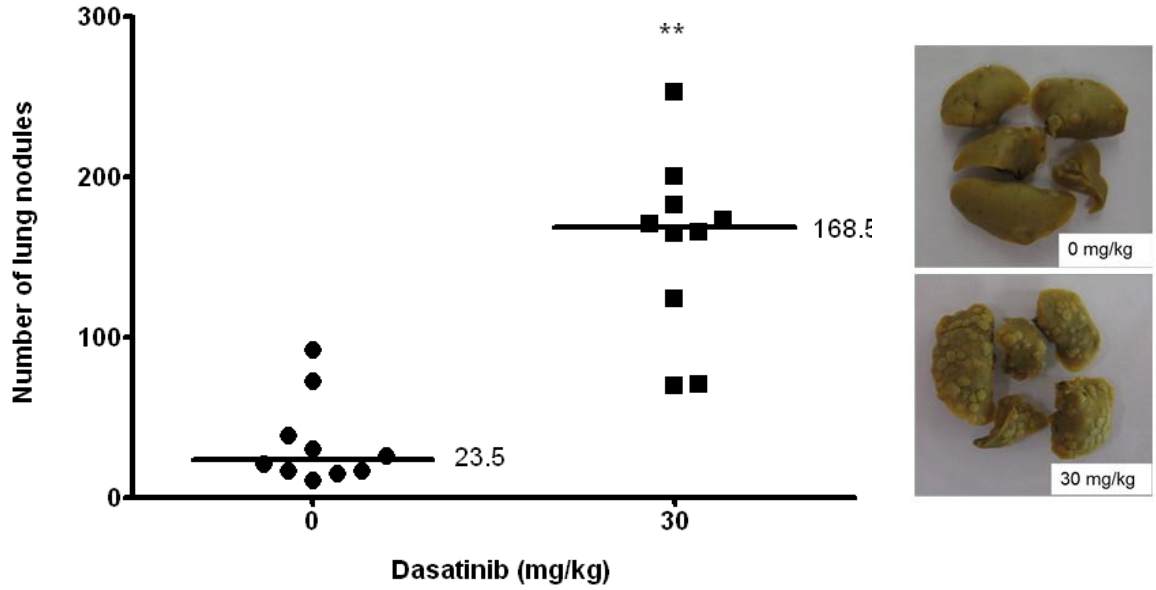

Figure 4-5. Lung metastasis assay: BALB/c mice injected with 10^5 4T1 cells. Mice were injected with 10^5 4T1 cells, and treated subsequently for five days with 30 mg/kg doses of Dasatinib. Each point on the graph represents the number of nodules found on a single set of lungs. Wilcoxon matched pairs test was used to analyze data in GraphPad Prism 5.0. ******$p<0.01$. Median values are shown.

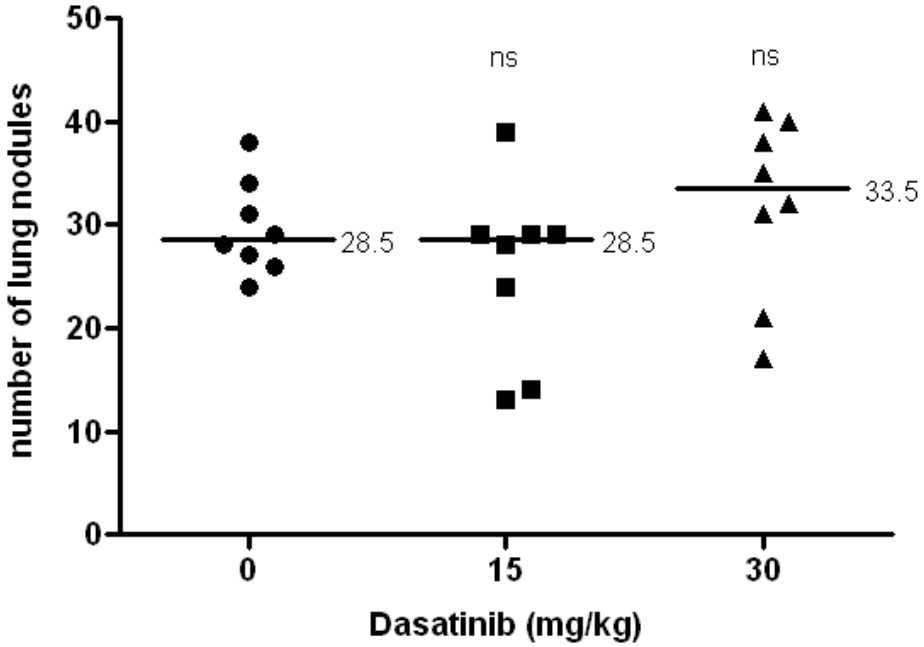

Figure 4-6. Lung metastasis assay: C3H mice. Mice were injected with $2x10^3$ KHT, and treated subsequently for five days with vehicle or various doses of Dasatinib. Each point on the graph represents the number of nodules found on a single set of lungs. Wilcoxon matched pairs test was used to analyze data in GraphPad Prism 5.0. Median values are shown.

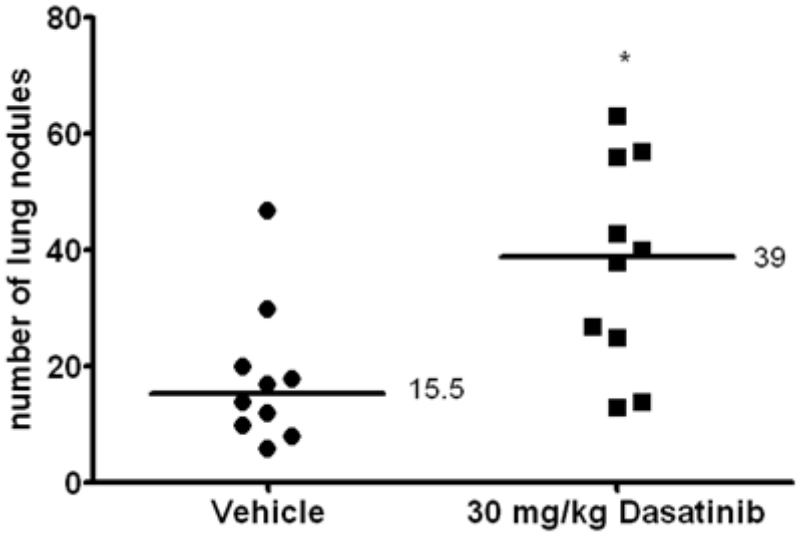

Figure 4-7. Lung metastasis assay: pretreated BALB/c mice. Mice were pretreated with vehicle or daily 30 mg/kg doses of Dasatinib for five days. On the sixth day, mice were injected with 10^5 4T1 cells. Each point on the graph represents the number of nodules found on a single set of lungs. Wilcoxon matched pairs test was used to analyze data in GraphPad Prism 5.0. *****$p<0.05$. Median values are shown.

CHAPTER 5
SUMMARY

Studies reported in this thesis have shown that: (i) Dasatinib potently inhibits migration and invasion *in vitro* at sub-cytotoxic doses, (ii) endothelial cell tube formation is not impaired by Dasatinib treatment, (iii) *in vitro* Dasatinib treatment confers radioresistance in the 4T1 tumor cell line, (iv) 4T1 tumor cells treated *in vitro* and then injected intradermally into mice are impaired in their ability to induce angiogenesis, (v) tumor cells treated *in vitro* with Dasatinib prior to inoculation into the blood stream of recipient hosts formed significantly fewer lung metastases than did untreated cells, (vi) 4T1 tumor cells injected intradermally into *in vivo* Dasatinib-treated mice are impaired in their ability to induce angiogenesis, and (vii) *in vivo* Dasatinib treatment enhances the ability of 4T1 tumor cells to form metastases when injected into the tail vein of mice.

4T1 tumor cells treated *in vitro* with Dasatinib were significantly impaired in their ability to migrate, invade, induce angiogenesis, and form lung metastasis, as expected. However, *in vitro* studies combining radiation revealed some unexpected findings. We combined Dasatinib with radiation therapy because new agents are typically combined with conventional therapy when being introduced into the clinic. Radiation is a very common treatment modality in many cancer types, including breast cancer. Since the therapy usually lasts several weeks, ideally it would be combined with an anti-metastatic agent to prevent metastases from forming for the treatment period. Much to our surprise, Dasatinib increased radiation resistance in the 4T1 cell line. The highest dose used (100 nM) increased survival by approximately a log. At the time, Dasatinib and radiation were combined in clinical trials for the treatment of head and neck and non-small cell lung cancers. We therefore chose to repeat the radiation experiment in those

cancer cell type lines. Furthermore we also examined several other cancer cell lines. All failed to recapitulate the radioprotective effect observed in the 4T1 breast cancer cells. The mechanism for this effect remains unknown but is unlikely due to Dasatinib influencing radiation damage.

A key question investigated in this thesis was: will Dasatinib inhibit metastasis *in vivo*? When mice injected with 4T1 tumor cells were treated with Dasatinib, the results were quite shocking and disconcerting. Mice treated with Dasatinib developed significantly higher numbers of lung metastases than vehicle-treated mice. With the belief that this may have been a cell-specific effect, or particular to the strain of mouse used, we repeated the experiment in a different cell line and mouse strain. The KHT fibrosarcoma cell line is syngeneic with the C3H mouse and has been extensively used in our laboratory to study the spread of cancer cells. It was chosen because previous investigations had demonstrated that another Src inhibitor (Saracatinib) successfully impaired the development of lung metastases in this model. However, Dasatinib did not inhibit lung colony formation *in vivo* in the KHT/C3H model. But, Dasatinib treatment also did not increase the formation of lung metastases in this model as had been observed in the 4T1 model. Given the results of these two experiments, we concluded that Dasatinib may be affecting to the host in some manner that might facilitate the establishment of secondary tumors in the lung tissue. In an initial attempt to test this possibility, mice were treated for five days with Dasatinib prior to introducing 4T1 tumor cells into the blood stream. A couple of control and treated mice were euthanized at the end of drug treatment prior to tumor cell injection and their lungs were taken for histological examination. The rest of the group was injected with tumor cells 24 hours

after the last drug dose, when most of the drug would have been cleared. The results showed that the Dasatinib pretreated mice developed significantly greater numbers of lung metastases, supporting our belief that Dasatinib was having an effect on the host, but unfortunately the histological assessments failed to demonstrate a direct effect of Dasatinib treatment on lung tissue. Additional studies will be required to ascertain how this agent is influencing the BALB/c host to lead to an enhanced development of 4T1 lung metastases.

These *in vivo* results were very surprising. There is a wealth of publications available demonstrating the efficacy of Dasatinib against invasion and migration *in vitro*. At the time of this writing, a search for the keywords “Dasatinib” and “metastasis” returned 3,110 hits on Google Scholar. But, out of those 3,110, only three demonstrated efficacy against *in vivo* metastasis when treating mice[28,29,30]. The first paper showed that Dasatinib inhibits lymph metastasis in a nude mouse model orthotopically injected with prostate cancer cells. Another paper demonstrated that Dasatinib reduces hepatic metastasis in a mouse model engineered to develop pancreatic adenocarcinoma. The final paper showed that Dasatinib treatment inhibited hepatic and lymph metastasis in a nude mouse model injected orthotopically with pancreatic adenocarcinoma cells.

In a culture where publishing positive results is easy, and negative results is very difficult, it is conceivable that other labs also failed to demonstrate *in vivo* efficacy with this agent. Should this project be followed up? Some will say because the drug does not work, it should be discarded and we should move onto one that does. However, this is more than a simple case of an ineffective drug. Under certain conditions, in at least one setting, this drug enhanced metastasis formation quite radically. There remains the

possibility that this agent may have a similar effect in (some) humans as it does in BALB/c mice. An estimate of the worldwide cumulative exposure to Dasatinib from its approval in 2006 till 2011 is 32,882 patients. This number is based on sales, and does not include those patients participating in clinical trials.

Dasatinib has been or currently is in phase II trials for the treatment of head and neck cancer, non-small cell lung cancer, squamous cell lung cancer, breast cancer, colorectal cancer, melanoma, prostate cancer, pancreatic cancer, glioblastoma, gliosarcoma, neuroblastoma, liver cancer, fallopian tube cancer, ovarian cancer, and gastrointestinal stromal cancer. Phase II trials typically include several hundred patients, so we can likely add a few thousand to the above number of exposed individuals. No studies have been done to observe the cause of death in long-term Dasatinib users.

Despite the results presented in this thesis, metastasis is still an important target in preventing cancer deaths. Over 90% of cancer-caused deaths are due to metastasis. A preventative agent could save the lives of many patients, particularly those with breast and prostate tumors. While Dasatinib may not have use in this setting, Src inhibitors as a group still hold value as potential anti-metastatic therapy.

Dasatinib and other Src inhibitors have been studied in clinical trials for solid tumors, and have done poorly. This should not come as a surprise. Clinical trials almost always measure surrogate endpoints such as tumor growth or time to progression. Clinical trials also use patients with advanced disease. Src inhibitors show little anti-growth activity, so should not be tested as an anti-growth agent. They will and do fail in this setting. Src inhibitors should be tested over a long period of time in patients who

have undetectable distant disease. However, this type of trial is time-consuming, costly, and very difficult to design, so such studies have as yet not been done.

In conclusion, Dasatinib does not inhibit metastasis *in vivo*. However, Src inhibitors as a group most likely hold value, but must be tested in early stage patients to demonstrate efficacy. Further research must be done to develop anti-metastatic therapies before a significant increase will be seen in quality of life and overall survival of cancer patients.

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BIOGRAPHICAL SKETCH

Veronica Saffran was born in Miami, Florida in 1988. She graduated from Martin County High School in 2006. In 2010, she graduated with honors from the University of Florida, receiving her Bachelor of Science degree in biology with a minor in chemistry. After graduation, she was accepted into the inaugural class of the Translational Biotechnology program at the University of Florida. This interdisciplinary program gave her the opportunity to earn a master's degree in medical science through coursework and reseach, while simultaneously earning a minor in business through various management, law, and marketing classes. In the fall of 2010, she joined the laboratory of Dr. Dietmar W. Siemann, where she spent two years with a primary research focus on metastasis. In the summer of 2012, she began an internship at Florida Biologix, a cGMP biopharmaceutical company. After graduation, she plans on pursuing oncology research or clinical trial management within a biotechnology company.