# The Effects of the Anti-Cancer Agent Dasatinib on KHT Murine Sarcoma Cells


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Metastatic sarcomas originate in connective tissues and often spread to secondary organs, such as the lungs. They prove significantly more difficult to treat once they have gained access to the circulatory system. This study assessed the efficacy of the molecular targeting agent dasatinib to inhibit the phosphorylation of Src, a non-receptor tyrosine kinase, and its downstream effector focal adhesion kinase (FAK). These proteins are often over-expressed in malignant cells and they mediate key cellular transduction pathways that contribute to metastatic activity. *In vitro* studies were done using a KHT murine sarcoma cell line to assess the impact of dasatinib on cellular growth, proliferation, migration, and invasion. Chemotherapeutic impact at the protein level was also studied. Treated cells demonstrated a dose-dependent decrease in phosphorylated-Src (pSrc) and phosphorylated-FAK (pFAK). Phenotypic changes included inhibition of proliferation, migration and invasion, and progression out of the G1 phase of cell cycle. Using immunofluorescence and standard biological assays, the Siemann lab has previously shown that the levels of pSrc and pFAK in a prostate cancer cell line decrease upon exposure to dasatinib. These findings come from literature reports of early clinical trials showing that dasatinib has promise as an anti-cancer agent. This warrants further studies to fully understand how it affects the metastatic cascade in particular.


## INTRODUCTION

Sarcomas are malignant growths that arise from various connective tissues in the body and can range in severity from low to high grade. There are approximately 50 different types that can occur anywhere in the body, with the most commonly diagnosed in the smooth muscles of the abdomen and the soft tissues of the legs. The survival rate of a patient diagnosed with sarcoma depends on the stage and grade of the tumor as well as other underlying factors, with earlier stages and lower grades resulting in a higher survival rate. While surgical excision is generally the primary method of treatment, adjuvant chemotherapy and radiation are frequently necessary. Despite the fact that sarcomas are rare compared to other cancers (10,660 new cases and 3,820 deaths in the U.S. last year) (American Cancer Society, 2009), a patient's prognosis and quality of life deteriorate rapidly if the tumor spreads to and invades secondary organ sites. The most prevalent secondary organs seen in metastatic sarcoma are the lungs, a diagnosis that consequentially drops the overall median survival down to only 15 months (Billingsley et al., 1999).

While research is ongoing, scientists have identified genetic factors as well as environmental factors and viruses that cause sarcoma. Tumor growth and proliferation occur when normal cellular onco-proteins are over-expressed (Pollock et al., 1997), proto-oncogenes are upregulated, or the cell fails to properly regulate growth factors (Todaro and De Larco, 1978). The proto-oncogene Src is a tyrosine kinase that is found in higher concentrations in tumor cells (Rosen et al., 1986) and is widely implicated in many aspects of tumorigenesis, invasion, and metastasis. One of the signal transduction molecules phosphorylated (activated) by Src is focal adhesion kinase (FAK), which is involved in cell adhesion and mobility and is also over-expressed in various types of cancers (Owens et al., 1995). Because of their fundamental roles in metastasis, Src and its downstream effector FAK are promising molecular targets. The anti-neoplastic agent dasatinib has multiple targets, including Src. It has already been approved by the FDA as a treatment for chronic myeloid leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia (ALL) in adults (National Cancer Institute, 2009). It is currently in phase II clinical trials for advanced sarcomas (clinicaltrials.gov).

The purpose of this study was to assess the efficacy of dasatinib in interfering with the cellular transduction pathways of Src, and subsequently those of FAK, resulting in the decreased ability of sarcoma cells to proliferate, invade, and metastasize. This would provide an alternative treatment strategy for sarcomas that are no longer responsive to existing therapeutic regimens as well as improve outcomes through local control.

## MATERIALS AND METHODS

### *Cell Culture and Reagents*

All experiments were done using a murine sarcoma KHT cell line (Rockwell and Kallman, 1972). The cells were maintained in Alpha modified minimum essential medium (αMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% 200-mmol/L ʟ-glutamine at 37 °C. They were collected by trypsonization. αMEM was used to de-trypsonize the cells, which were then centrifuged for 10 min. at 1000rpm. All reagents used were obtained from Life Technologies. Primary antibodies for immunofluorescence for pSrc were from Cell Signaling Technologies and for pFAK were from BioSource International. Secondary antibodies were from Invitrogen.

### *Drug Preparation*

Dasatinib was provided by Bristol-Myers Squibb Pharmaceutical Research Institute. A 10mM stock solution, prepared in dimethyl sulfoxide, was diluted to the appropriate concentration using phosphate buffered saline (PBS). The drug was kept frozen and prepared immediately before use at a dilution of 10ul/mL drug to media.

### In Vitro *Cell Growth*

KHT cells were plated in triplicate at $10^4$ in 60mm cell culture dishes. After 24 hours, the medium was then replaced and treated with dasatinib (0.1uM, 0.5uM, 1.0uM, 5.0uM, or 10uM). The cells were collected and counted using trypan blue staining 1, 2, 4, and 7 days after treatment. Adhered and floating cells were harvested and counted separately to account for the high numbers of detached cells.

### *Migration Assay*

Appropriate concentrations of dasatinib (0.1uM, 0.5uM, 1.0uM, 5.0uM, or 10uM) were added to the top and bottom wells of modified Boyden migration chambers (BD Falcon) with 8μm pore size. KHT cells were then plated in the top well at $10^3$ cells/200mL and kept at 37 °C for 48 hours. The inside of the upper wells were scraped and then stained with crystal violet dye. The number of cells that had migrated through the membrane was counted under a dissecting microscope.

### *Invasion Assay*

αMEM media with 0.1% FBS was used to rehydrate a BD BioCoat Matrigel Invasion Chamber for 2 hours at 37 °C. $2x10^4$ cells treated with dasatinib (0.1uM, 0.5uM, 1.0uM, 5.0uM, or 10uM) in 200 mL of the same media were added to the top wells. αMEM with 10% FBS was added to the bottom chamber. After 48-hour incubation at 37 °C, the insides of the upper wells were scraped and then stained with crystal violet. Cells were then counted using a dissecting microscope.

### *Cell Cycle Analysis*

Subconfluent KHT cells were treated with dasatinib (0.1uM, 0.5uM, 1.0uM, 5.0uM, and 10uM) and incubated at 37 °C for 24 hours. They were collected using the previously stated protocol and prepared at $10^6$ cells/3mL in a solution of 50% PBS and 50% cold 100% ethanol. Cells were then stored at 4 °C overnight. Cells were centrifuged and then treated with 1mg/ml RNase-A for 30 min. with intermittent vortexing, after which they were spun for 10min. at 4 °C at 1000rpm. Cells were stained with propidium iodide and analyzed using a FACScan flow cytometer, then analyzed using ModFitLT 3.0 software.

### *Immunofluorescence and Confocal Microscopy*

Cells were plated at $2x10^4$ cells/mL in 35mm tissue culture dishes (Fluorodish™) and treated with 10μM dasatinib after 24 hours. They were fixed with 3% formaldehyde in 1x Cytoskeleton Buffer (1mL 10X CB, 320mM Sucrose, 2mM EGTA, brought to volume with $dH_2O$) (per 100mL of 10X CB: 1.95g MES, 0.285g $MgCl_2$, and 10.29g KCl, brought to volume with $dH_2O$). Primary and secondary antibodies for pSrc and pFAK were administered according to company protocols. The cells were fixed with Vectashield mounting medium with DAPI to stain nuclear DNA. Imaging was done using a confocal microscope at 64X magnification.

## RESULTS

### *Dasatinib Inhibits Cell Growth* In Vitro

Dasatinib inhibited KHT growth in a dose-dependent fashion with results beginning 48 hours after treatment (Figure 1).

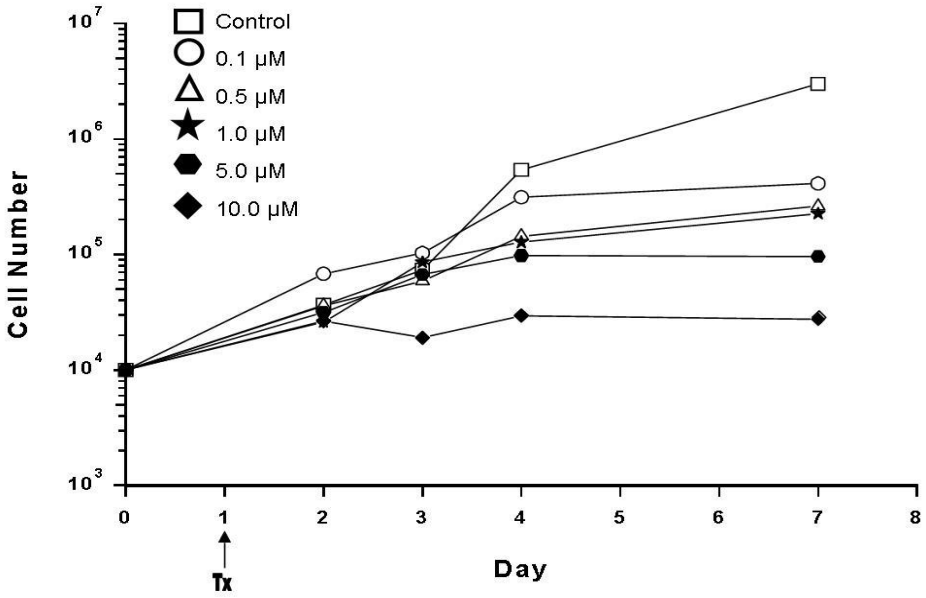


**Figure 1:** Growth curve of sarcoma cells after treatment with dasatinib. Tx indicates treatment, which was administered 24 hours after cells were plated. Proliferation decreases as dosage increases, with clear effects at the 4 day point.

### *Dasatinib Inhibits KHT Migration*

KHT cells were treated with dasatinib; the number of cells that had migrated across a Matrigel membrane was counted after staining with crystal violet. The number of cells per dosage was determined by averaging three wells. Figure 2 is the average of 4 experiments. Migratory ability was inhibited in a dose-dependent fashion. This inhibition was significant ($p < .01$) beginning with the 0.5μM treatment (Figure 2).

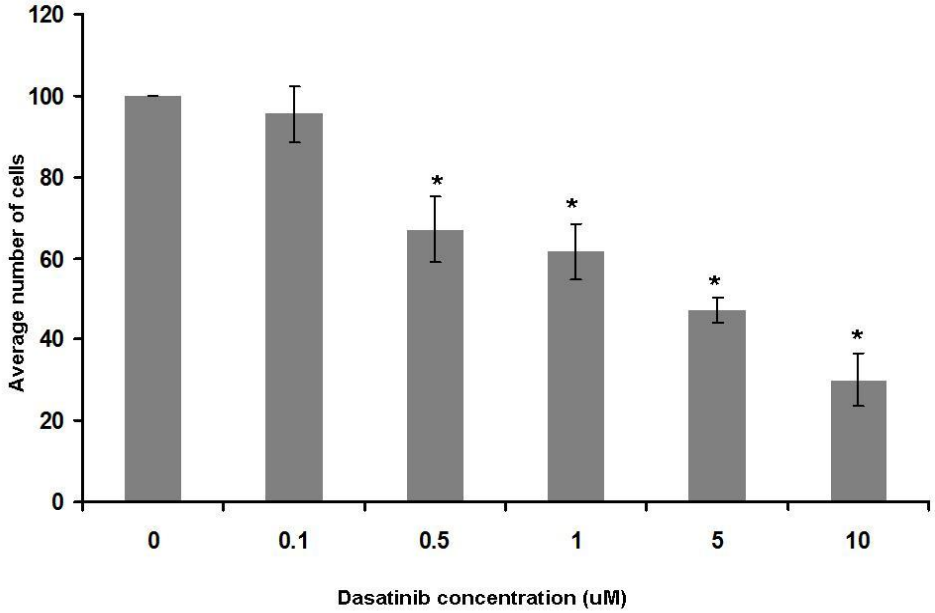


**Figure 2:** Dasatinib inhibits migration. Increased concentrations of the drug resulted in a decrease in the average number of cells able to cross the membrane. * indicates significance, with $p$ <0.01.

### *Dasatinib Inhibits KHT Invasion*

Exposure to dasatinib resulted in a dose-dependent decrease in the amount of KHT cells able to invade the Matrigel and cross the membrane. As seen in the migration, this inhibition was significant beginning with the 0.5µM treatment (Figure 3).

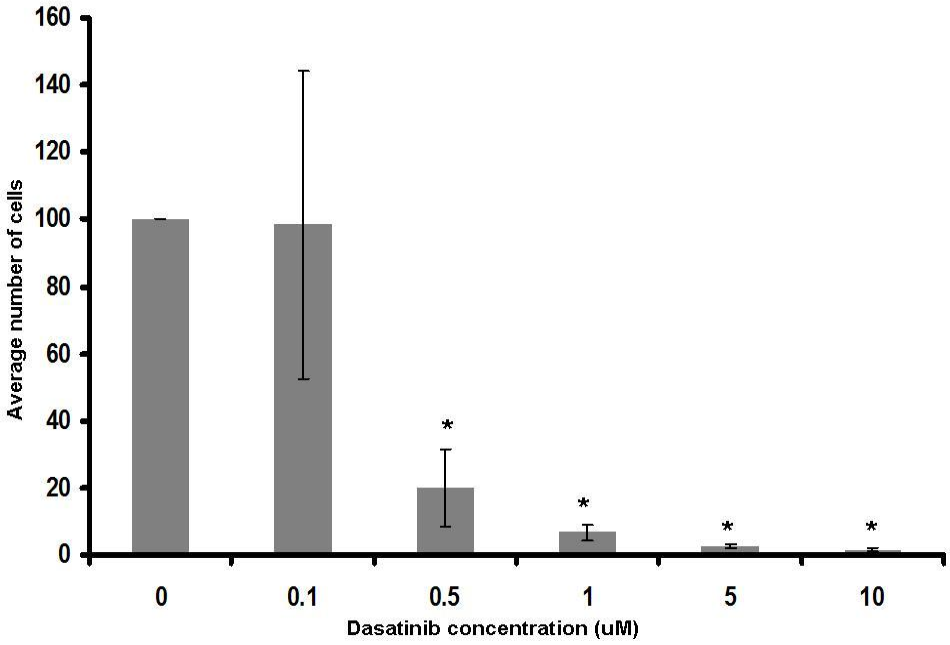


**Figure 3:** Dasatinib inhibits invasion. Increased concentrations of drug resulted in a significant decrease in the average number of cells able to invade the Matrigel. ***** indicates significance, with $p < 0.01$.

### *Dasatinib Treatment Results in an Accumulation at the G1 Phase of the Cell Cycle*

The number of KHT cells that accumulated in the G1 phase of the cell cycle was greater after dasatinib treatment, relative to controls, with a concomitant decrease in S and G2/M phases (Table 1). These cell cycle trends were dose dependent. The accumulation of cells in the G1 phase is evident in the DNA histograms in Figure 4, with the 1.0µM treatment resulting in a greater than twofold increase in G1 phase than in the control.

**Table 1:** Amount of DNA in Different Stages of the Cell Cycle Determined Using Flow Cytometry

| Dasatinib (µM) | G1 Phase (%) | G2/M Phase (%) | S Phase (%) |
|---|---|---|---|
| Control (0) | 38.4 | 18.0 | 43.6 |
| 0.1 | 54.0 | 14.6 | 31.3 |
| 0.5 | 80.9 | 7.4 | 11.7 |
| 1.0 | 85.5 | 5.6 | 8.9 |

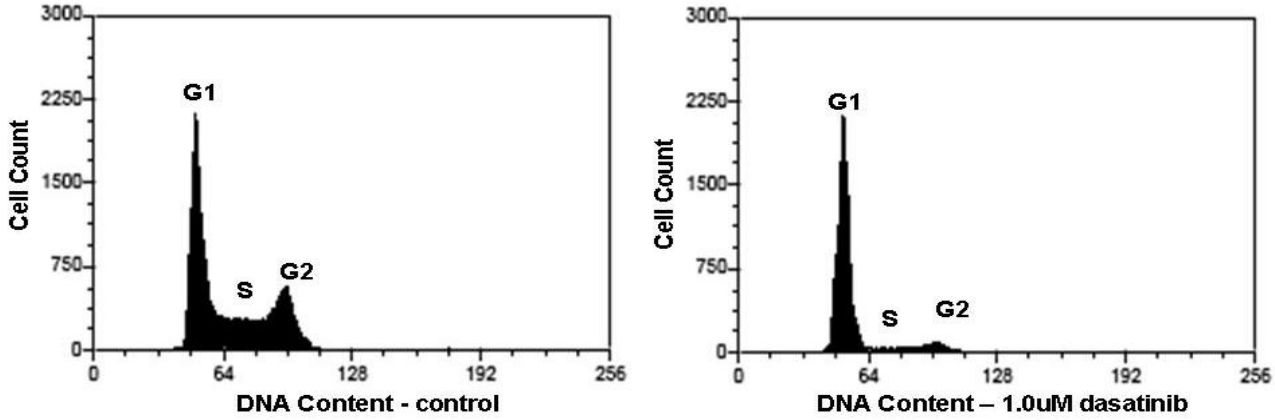


**Figure 4:** Cell cycle analysis showing the DNA distribution in control and 1.0µM dose cells. Shown here are representative histograms. Exposure to dasatinib resulted in cell accumulation in the G1 phase. There are fewer cells in the S and G2 phases seen in the 1.0µM dasatinib histogram.

### *Dasatinib Decreased pSrc and pFAK at Focal Adhesions Sites*

The control KHT cells showed pSrc localized at the periphery of the cell where the focal adhesion sites would be found (Figure 5A). In contrast, pSrc was delocalized throughout the cytoplasm in cells exposed to dasatinib (Figure 5B). The same results were demonstrated in cellular pFAK distribution (Figures 6A, 6B).

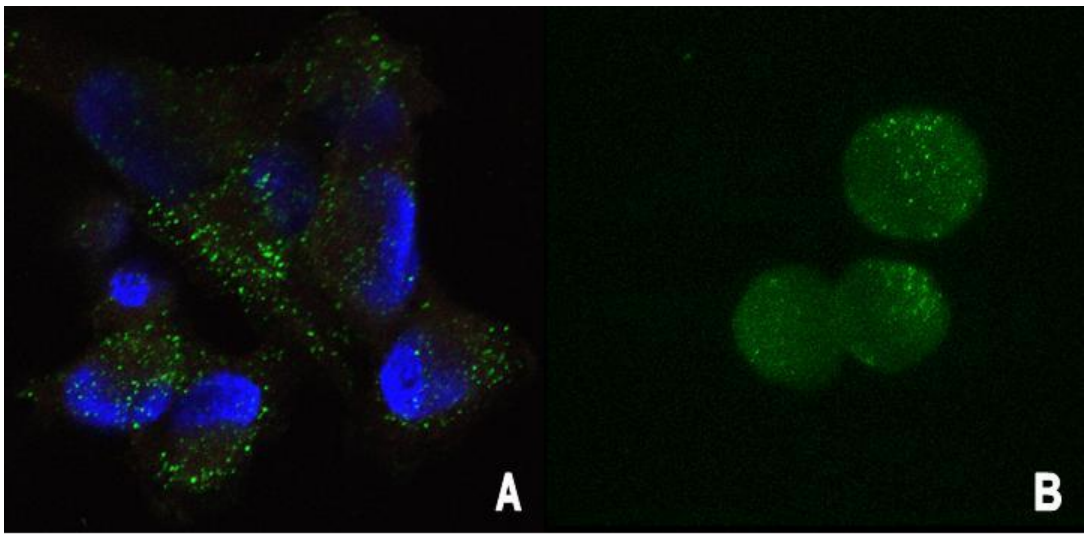


**Figure 5:** Dasatinib decreased the amount of phosphorylated-Src at focal adhesion sites. (A) shows control phosphorylated-Src (bright green) localized around the cell membranes. Nuclei are stained blue. (B) is a higher magnification showing phosphorylated-Src redistributed throughout the cytosol of a cell after exposure to 1.0µM dasatinib.

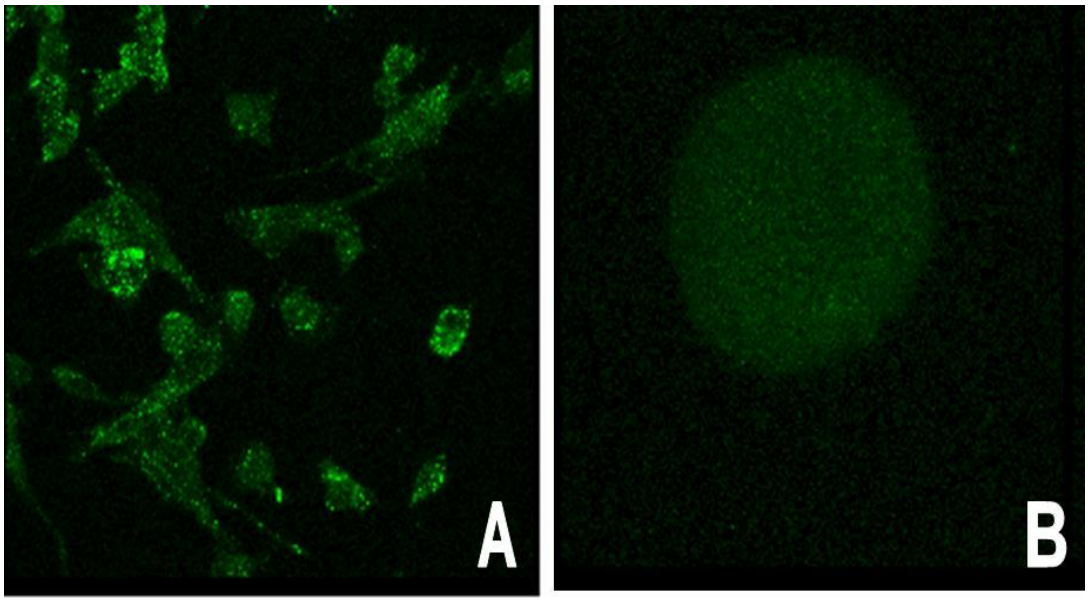


**Figure 6:** Dasatinib decreased the amount of phosphorylated-FAK at focal adhesion sites. (A) shows control phosphorylated-FAK (bright green) localized around the cell membranes. (B) is a higher magnification of a single cell showing phosphorylated-FAK redistributed throughout the cytosol of a cell after exposure to 1.0μM dasatinib.

## DISCUSSION

Metastatic potential in sarcoma cells is largely attributed to anomalies in the signaling pathways regulated by the Src family kinases (Thomas & Brugge, 2007). Studies have confirmed elevated levels of Src as well as its downstream effector FAK in various malignant cell lines, including those of sarcoma (Rosen et al., 1986). Inhibiting the phosphorylation of Src and FAK using dasatinib would hinder pathways responsible for KHT cell growth, migration and invasion. Dasatinib is in phase II clinical trials to treat advanced stage sarcoma, but there is not extensive research focusing on its ability to prevent metastasis in particular.

KHT sarcoma cells grow rapidly, which enables the growth and development of tumors. Dasatinib effectively inhibited *in vitro* cell growth in a dose-dependent manner. These results are consistent with those found in pancreatic cell lines (Chang et al., 2008); however, Buettner et al. (2008) found that dasatinib did not strongly affect proliferation in melanoma cells. The ability of dasatinib to inhibit KHT growth is corroborated by cell cycle analysis in which treated cells accumulated in the G1 phase. The decrease in amount of DNA per cell undergoing replication in the S phase would help explain the inhibition observed in the growth curve. Various studies using inhibitory antibodies and agents that arrest cell cycle have shown the importance of Src in cell progression through the S and G2 phases into mitosis (Mamidipudi et al., 2004; Roche et al., 1995).

There are multiple regulatory mechanisms for Src tyrosine kinase. It is often activated by more than one of these regulation mechanisms (one of which is phosphorylation), and then goes on to activate growth factor receptor proteins and mitotic kinase pathways such as the Ras-MAP pathway (Thomas & Brugge, 2007). FAK is also involved in growth and proliferation through its interactions with growth factors and integrins.

Dasatinib is known to effectively inhibit migration and invasion in several cancer models, and this study confirms this in the KHT sarcoma cell line. These results were further verified by immunofluorescence. Focal adhesion kinase is recruited to focal adhesion sites by integrins, where it interacts with actin to cause a turnover of these sites that propels the cell along the substrate. The auto-phosphorylation of FAK attracts activated pSrc, which then phosphorylates it at three tyrosine residues (Parsons, 2003). Sieg et al. (1999) found that Fak-deficient fibroblast mutants demonstrate retardation in migratory ability as well as morphological defects. Less pSrc and pFAK at the focal adhesion sites of cells after treatment (seen in Figure 5 and 6) help to explain the inhibition of migration and invasion and emphasizes the importance of Src as an upstream activator of FAK. Additionally, the Siemann lab has used protein immunoblots to demonstrate decreased protein levels of activated Src and FAK after dasatinib

treatment in other cancer cell lines. Shor et al. (2007) found that dasatinib also inhibits migration and invasion in various human sarcoma cell lines.

Preventing the spread of cancer is crucial to patient survival, mainly because treatment options are limited after secondary organ involvement. These experiments show that the anti-cancer drug dasatinib is a potent inhibitor of murine sarcoma cell migration and invasion, which are key components of metastasis. Consistent with related research, it also inhibits proliferation and causes these cells to accumulate in the G1 phase of the cell cycle. Further research on the efficacy of this agent in preventing metastasis in animal models is needed. However, dasatinib has the potential to treat fibroblast sarcoma as well as prevent tumor formation in secondary organs.

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