Src Kinase Inhibitors for the Treatment of Sarcomas:

Cellular and Molecular Mechanisms of Action

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of
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Dedication

I dedicate this to my father, Richard C. Burns for his love and devotion – inspiring my yearning to seek more.

And to my wonderful, caring husband Adam. For his endless love and support throughout our journey.
Acknowledgements

I would like to acknowledge Sean Yoder and the rest of the members of the H. Lee Moffitt Microarray Core for completing the microarrays and aiding in the analysis. I would also like to graciously thank Samuel Falsetti for the coffee breaks and brainstorming sessions throughout all of our hurdles. Ralf Buettner for helping me get started in the beginning, laying the foundation and molding me into the scientist I am today. Bonnie Goodwin, for being there for me during the most difficult year of my life. Tania Mesa for keeping me sane with our long runs every weekend during the final stages. Lastly, I would like to thank my wonderful mentors, Drs. Rich Jove and Jack Pledger for having faith in me, Drs. Doug Letson and Carlos Muro-Cacho for their constant support.
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List of Abbreviations

AJ  Adherens Junctions
AS  Adenosarcoma
ATP Adenosine Triphosphate
CAS Crk and Src Associated Substrate
Chk Csk homologous kinase
CS  Chondrosarcoma
DFSP Dermatofibrosarcoma Protuberans
DMSO Dimethyl sulfoxide
EGFR Epidermal Growth Factor Receptor
EMSA Electrophoretic Mobility Shift Assay
EWS Ewing’s sarcoma
FAF1 Fas Associated Factor 1
FAK Focal Adhesion Kinase
FBS Fibrosarcoma
Fig Figure
GEP Gene Expression Profile
GIST Gastrointestinal Stromal Tumor
h  Hour
HSP Heat Shock Protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% Inhibitory Concentration</td>
</tr>
<tr>
<td>ICD</td>
<td>International Classification of Diseases</td>
</tr>
<tr>
<td>IGFR-1</td>
<td>Insulin-like Growth Factor Receptor-1</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LMS</td>
<td>Leiomyosarcoma</td>
</tr>
<tr>
<td>LPS</td>
<td>Liposarcoma</td>
</tr>
<tr>
<td>MAS</td>
<td>Affymetrix Microarray Suite</td>
</tr>
<tr>
<td>MHF</td>
<td>Malignant Fibrous Histiocytoma</td>
</tr>
<tr>
<td>MFS</td>
<td>Myxoid Fibrosarcoma</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromatosis type 1</td>
</tr>
<tr>
<td>N/R</td>
<td>No Response</td>
</tr>
<tr>
<td>NRTK</td>
<td>Non-receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>OSA</td>
<td>Osteosarcoma</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet Derived Growth Factor Receptor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RD</td>
<td>Rhabdomyosarcoma</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SAM</td>
<td>Statistical Analysis of Microarrays</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SCS</td>
<td>Synovial Cell Sarcoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SFK</td>
<td>Src Family Kinase</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology</td>
</tr>
<tr>
<td>siRNA</td>
<td>Silencing RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signaling and Transducer of Activation Transcription</td>
</tr>
<tr>
<td>STS</td>
<td>Soft Tissue Sarcoma</td>
</tr>
<tr>
<td>TK</td>
<td>Tyrosine Kinase</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine Kinase Inhibitor</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>Y</td>
<td>Ty</td>
</tr>
</tbody>
</table>
Sarcomas are rare mesenchymally-derived tumors with limited treatment options. Tyrosine kinases may serve as potential targets for sarcoma therapy because many are mutated or overexpressed in sarcomas and cell lines. One potential molecular target for sarcoma treatment is the Src tyrosine kinase. Three independently synthesized Src kinase inhibitors were evaluated in human sarcoma cell lines. Of the three, dasatinib, provided promising results as a potential sarcoma therapy. Until this study, dasatinib activity had not been characterized in sarcoma cells. Based on our previous findings of Src activation in human sarcomas, we evaluated the effects of dasatinib in twelve sarcoma cell lines. Dasatinib inhibited Src activity and downstream signaling at nanomolar concentrations. Inhibition of Src signaling was accompanied by blockade of cell migration and invasion. Moreover, apoptosis was induced in a subset of bone sarcomas at nanomolar concentrations of dasatinib. Inhibition of Src protein expression by siRNA also induced apoptosis, indicating that these bone sarcoma cell lines are dependent on Src activity for survival. These results demonstrate that dasatinib inhibits migration and invasion of diverse sarcoma cell types, and selectively blocks the survival of bone sarcoma cells.
Therefore dasatinib may provide therapeutic benefit by preventing the growth and metastasis of sarcomas.

Microarray analysis of the sarcoma cell lines lead to the identification of a molecular signature that successfully predicts response to dasatinib by induction of apoptosis. Components of this molecular signature are expressed in primary human sarcomas. Furthermore, expression of the molecular signature in sarcomas can be utilized to cluster tumors based on theoretical response to dasatinib. While the prediction of response in tumors is theoretical, there is encouraging evidence to support further endeavors into validating the potential of this molecular signature to predict response in patients.

Together, these studies reveal that, in cell lines, both constitutive Src activation and the presence of a molecular signature that predicts response to dasatinib are important parameters to consider when selecting dasatinib as a treatment for. Furthermore, novel therapeutic approaches that inhibit Src signaling may selectively induce apoptosis in tumor cells and sensitize to chemotherapy those tumors that contain the relevant molecular signature.
Introduction

Epidemiology of sarcomas

Sarcomas comprise a relatively rare and diverse group of malignant tumors that arise from mesenchymally-derived connective tissues including bone, fat and muscle. There are more than 50 different subtypes of sarcomas, with approximately 12,000 new cases diagnosed nationwide each year (1, 2). While this represents a fraction of all cancers diagnosed in the United States (3), sarcomas account for approximately 20% of newly diagnosed pediatric solid tumor malignancies (4) and are among the cancers that pose the greatest risks of mortality and morbidity in children and young adults (5-9). Histologically, sarcomas are divided into two sub-categories, soft-tissue and bone sarcomas. There are over 9,000 cases of soft tissue sarcomas expected to be diagnosed in the United States this year and 5,500 Americans are expected to die as a result this disease (10). Less than 1,000 soft tissue sarcomas are diagnosed in children each year in the United States, with an annual incidence of approximately 11 per million (11). The most commonly diagnosed soft tissue sarcomas (STS) include malignant fibrous histiocytomas (MFH), liposarcomas (LPS) and leiomyosarcomas (LMS) (Table 1) (10-12). In 2006, over 2,760 new cases of bone sarcoma are expected to be diagnosed in the United States and 1,260 Americans are expected to die from complications from these tumors (13). Bone sarcomas may occur at any age and account for less than 0.2% of all cancers diagnosed each year, with 30% observed in children and young adults (13).
Osteosarcoma (OSA) is the most commonly diagnosed primary bone tumor followed by Ewing’s sarcoma (EWS), chondrosarcoma (CS) and fibrosarcoma (FBS) (13, 14).

In the United States, sarcomas affect men more often than women, and white men have the highest overall incidence among all citizens (Table 2) (4,17). There is a high frequency of sarcomas reported in Uganda and Zimbabwe which is related to the high prevalence of HIV/AIDS infections in those countries (18). Israel has the highest prevalence of all non-African nations with an elevated age-standardized incidence of diagnoses that is consistent with previous reports of increased incidence of sarcomas in men of Jewish background (19, 20).

The detection of specific molecular abnormalities has contributed significantly to sarcoma classification while eliciting an interest in targeted therapies (1). Several well-described genetic mutations that predispose individuals to specific types of sarcomas have been described. The most common are mutations in the retinoblastoma (Rb) pathway, which increase the risk of developing osteogenic sarcomas (21), Li-Fraumeni syndrome, which is caused by a p53 mutation, increases the risk of a variety of sarcomas (22-25), deletion of the neurofibromatosis type 1 (NF1) gene increases the risk of malignant nerve sheath tumors (26) and c-kit gene mutations are responsible for the development of gastrointestinal stromal tumors (GIST) (27).
**Table 1:** Relative frequency of subtypes of sarcomas

<table>
<thead>
<tr>
<th>Histological Subtypes</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant fibrous histiocytoma</td>
<td>28.15</td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>15.16</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>11.94</td>
</tr>
<tr>
<td>Synovial sarcoma</td>
<td>10.08</td>
</tr>
<tr>
<td>Malignant peripheral nerve sheath tumor</td>
<td>5.81</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>4.84</td>
</tr>
<tr>
<td>Unclassified sarcoma</td>
<td>11.29</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>3.06</td>
</tr>
<tr>
<td>Ewing’s sarcoma</td>
<td>2.02</td>
</tr>
<tr>
<td>Angiosarcoma</td>
<td>2.02</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>1.13</td>
</tr>
<tr>
<td>Epithelioid sarcoma</td>
<td>1.13</td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>1.05</td>
</tr>
<tr>
<td>Clear cell sarcoma</td>
<td>0.97</td>
</tr>
<tr>
<td>Alveolar soft part sarcoma</td>
<td>0.56</td>
</tr>
<tr>
<td>Malignant hemangiopericytoma</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Adapted from (15)

**Table 2:** Incidence of soft tissue sarcomas diagnosed in the US by race, SEER, 1998-2002

<table>
<thead>
<tr>
<th>Race/Ethnicity</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Races</td>
<td>3.5 per 100,000 men</td>
<td>2.4 per 100,000 women</td>
</tr>
<tr>
<td>White</td>
<td>3.6 per 100,000 men</td>
<td>2.4 per 100,000 women</td>
</tr>
<tr>
<td>Black</td>
<td>3.5 per 100,000 men</td>
<td>2.6 per 100,000 women</td>
</tr>
<tr>
<td>Asian/Pacific Islander</td>
<td>2.7 per 100,000 men</td>
<td>1.6 per 100,000 women</td>
</tr>
<tr>
<td>Hispanic</td>
<td>3.0 per 100,000 men</td>
<td>2.3 per 100,000 women</td>
</tr>
</tbody>
</table>

Adapted from (16)
Risk factors investigated for the development of sarcomas

Few risk factors have been associated in the literature with the development of sarcomas (reviewed in Table 3). Epidemiologic studies have identified radiation, certain chemotherapies and vinyl chloride as significant factors while data is conflicting with regard to pesticides and herbicides. Currently, phenoxyacetic acid and chlorophenol have been accepted as causal agents in the development of sarcomas, although the data generated from epidemiologic studies investigating this association do not support this conclusion, (28-38). Dioxin and Agent Orange, more potent forms of pesticides and herbicides, have not been shown to play a significant role in the induction of sarcomas (39-49). There have been no published reports suggesting an association between viruses and the development of sarcomas in humans to date although ongoing investigations are trying to better understand the role of viruses in sarcomagenesis (50, 51).

The relationship between traumatisms, burns and scars and sarcomagenesis is weak. Case series and isolated reports are the only studies available to assess the relevance of these risk factors and a measure of the magnitude of an association has not been established so far. Even in case-control studies a temporal relationship is difficult to establish. Nevertheless, there have been numerous reports of sarcoma development in the location of previous injuries. However the lack of objective quantitation and the uncertainty in the temporal sequence of events has prevented the establishment of a definitive exposure-outcome.

Many difficulties plague epidemiological studies of sarcoma. One example is the previous faults inherent of the International Classification of Diseases (ICD) system. The
Identification of sarcomas was complicated by inefficient coding rules, which until recently, classified sarcomas according to visceral organ or connective tissue of origin, rather than placing sarcomas in one specific ICD category. As a result, many sarcomas were not accurately reported and this has complicated retrospective analyses. It is expected that recent improvements in the methodology will improve recognition.

Another concern of epidemiology studies has been the poor correlation between the diagnoses recorded on death certificates and hospital records.

The rarity of sarcomas leads to a greater chance of misdiagnosis and misclassification. This is complicated by discordant diagnostic opinions even among experts. Furthermore, methodological deficiencies in study design to investigate potential risk factors have been common. An example is the difficulty in determining a precise exposure to a risk factor and, often, surrogate measurements are used to estimate average exposures. Also, in the case of pesticides and herbicides, it has been difficult to precisely determine the exact chemical composition of the substances of interest in a given cocktail. Thus, it is speculated that the observed risk from exposure to phenoxy herbicides may become insignificant when controlling for some of these concomitant exposures. Thus, further studies are needed to establish a relationship between exposure to phenoxyacetic acids and chlorophenols and development of sarcomas. The known epidemiologic risk factors of sarcomagenesis have had major public health implications but more research is still needed. Sarcoma is an extremely rare cancer and there are insufficient cases to conduct valid, classic epidemiologic studies.
Table 3: Potential risk factors evaluated by epidemiological studies.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Type of Study</th>
<th>Conclusion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Radiation</strong></td>
<td>Case-series and Case-reports</td>
<td>Definitive</td>
<td>(38, 52-76) (77-80)</td>
</tr>
<tr>
<td>Thorotrust</td>
<td>Case-control</td>
<td>Probable</td>
<td>(81-84)</td>
</tr>
<tr>
<td>Breast Conservation Therapy</td>
<td>Case-series and Case-reports</td>
<td>Probable</td>
<td>(85, 86)</td>
</tr>
<tr>
<td><strong>Chemotherapy</strong></td>
<td>Retrospective cohort</td>
<td>Definitive</td>
<td>(87)</td>
</tr>
<tr>
<td></td>
<td>Case-series</td>
<td>Probable</td>
<td>(88)</td>
</tr>
<tr>
<td></td>
<td>Case-control</td>
<td>Probable</td>
<td>(73)</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>Case-series</td>
<td>Possible</td>
<td>(89-92)</td>
</tr>
<tr>
<td><strong>Vinyl Chloride</strong></td>
<td>Retrospective cohort</td>
<td>Definitive</td>
<td>(93-97)</td>
</tr>
<tr>
<td></td>
<td>Retrospective cohort</td>
<td>Unclear</td>
<td>(98)</td>
</tr>
<tr>
<td></td>
<td>Case-series</td>
<td>Probable</td>
<td>(99)</td>
</tr>
<tr>
<td><strong>Pesticides &amp; Herbicides</strong></td>
<td>Retrospective cohort</td>
<td>No Association</td>
<td>(34, 37, 38)</td>
</tr>
<tr>
<td></td>
<td>Case-control</td>
<td>No Association</td>
<td>(32, 36)</td>
</tr>
<tr>
<td></td>
<td>Retrospective cohort</td>
<td>Possible</td>
<td>(35)</td>
</tr>
<tr>
<td></td>
<td>Case-Control</td>
<td>Possible</td>
<td>(31, 33)</td>
</tr>
<tr>
<td></td>
<td>Occupational Study</td>
<td>Possible</td>
<td>(28-30)</td>
</tr>
<tr>
<td><strong>Dioxin</strong></td>
<td>Retrospective cohort</td>
<td>Possible</td>
<td>(41, 44)</td>
</tr>
<tr>
<td></td>
<td>Prospective cohort</td>
<td>No Association</td>
<td>(40)</td>
</tr>
<tr>
<td><strong>Agent Orange</strong></td>
<td>Case-control</td>
<td>No Association</td>
<td>(39, 42, 43, 45-49)</td>
</tr>
<tr>
<td><strong>Lymphadema</strong></td>
<td>Case-report</td>
<td>Possible</td>
<td>(100)</td>
</tr>
<tr>
<td></td>
<td>Case-series</td>
<td>Possible</td>
<td>(101)</td>
</tr>
<tr>
<td><strong>Trauma</strong></td>
<td>Case-series</td>
<td>Possible</td>
<td>(102)</td>
</tr>
<tr>
<td></td>
<td>Case-control</td>
<td>Possible</td>
<td>(103)</td>
</tr>
<tr>
<td><strong>Foreign Body Implantation</strong></td>
<td>Case-report</td>
<td>Probable</td>
<td>(104-111)</td>
</tr>
<tr>
<td><strong>Burns &amp; Surgical Scars</strong></td>
<td>Case-series</td>
<td>Possible</td>
<td>(112-119)</td>
</tr>
<tr>
<td><strong>Viruses</strong></td>
<td>Retrospective cohort</td>
<td>No Association</td>
<td>(50, 51)</td>
</tr>
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</table>
A molecular epidemiology approach guided by classic principles may provide a more appropriate venue. The data reported in the literature supports causal roles for radiation, vinyl chloride, certain chemotherapies and pesticides and herbicides containing phenoxyacetic acid and chlorophenol exposures, as well as previous cases of lymphedema. This evidence has restricted occupational exposures to vinyl chloride, pest- and herbicides. Radiation therapy has been re-evaluated and lesser doses are currently used to try to prevent the occurrence of future sarcomas. Physicians are monitoring patients who are receiving treatments such as tamoxifen in order to detect adverse effects before a sarcoma develops. Furthermore, the problems evident in the ICD coding of sarcomas have prompted a more sophisticated method of classifying rare diseases. This will aid in resolving the difficulties in detecting each case and allow for more thorough and complete studies of rare disease such as sarcomas in the future.

There have been recent advances in the understanding of sarcoma molecular biology. Employing what is currently known of the epidemiologic risk factors and the knowledge gained at the bench will help tailor design future studies. These studies should correlate environmental exposures with the molecular biology of the disease. Collectively, epidemiologists and basic scientists can achieve a more thorough understanding of sarcomagenesis. Together with a more efficient classification system, this will provide a more precise approach in the design of future clinical trials and treatments, as well as a greater understanding of potential risk factors of sarcomas yet to be identified.
Limitations of current treatment options

The primary approach to treating sarcomas is surgery. Radiation therapy is also used following surgery for patients with unresectable or residual tumors. Systemic chemotherapy, such as doxorubicin and ifosphamide are used to treat patients with metastatic disease. Pediatric and certain cases of chemosensitive sarcomas undergo chemotherapy treatment following surgery and/or radiation treatment as an effort to prevent recurrence or metastasis. However, because many sarcomas harbor Rb mutations and exposure to radiation increases the risk of developing sarcomas, variations in the method of treatment is required to prevent further complications. While this approach has proven effective in treating patients with localized disease, in many cases only modest degrees of success are achieved. Furthermore, treatment for patients with advanced and/or metastatic disease remains limited and chemotherapy is palliative in nature at best.

Doxorubicin was first employed as a treatment option for metastatic soft tissue sarcomas in 1972 at MD Anderson, Houston, TX (120) and ifosphamide was introduced in the late 1980s (121). The outcome for most adult patients with advanced sarcoma still remained bleak, however. Recently, the power of molecular biology was clearly demonstrated with the introduction of Gleevec as a treatment option for GISTs (122, 123). Revealing a new method of approaching sarcoma treatment. To fully appreciate this, the role of signal transduction in cancer and most specifically in sarcomas must first be reviewed.
Role of signal transduction in cancer

Protein phosphorylation plays a key role in nearly every aspect of cellular molecular biology. It is controlled by kinases, which phosphorylate, and phosphates, which de-phosphorylate protein-serine, -threonine and -tyrosine residues. To date, 90 tyrosine kinases - 58 transmembrane receptors and 32 non-receptor proteins (124) and 107 protein-tyrosine phosphatase genes (125) have been identified in the human genome. Signals are communicated in the cell either through external or internal stimuli. External stimulation is elicited through cell receptors and internal stimulation is processed through non-receptors tyrosine kinases which activate specific proteins and transmit the signal to other proteins within the cell, ultimately transducing the signal to the nucleus, regulating gene transcription. Cancer arises as a result of aberrant activation or stimulation of signal transduction pathways.

Genetically, sarcomas fall into two subgroups, those with complex karyotypes characteristic of severe genomic instability and those characterized by near-diploid karyotypes. Sarcomas with simple, near-diploid karyotypes usually possess specific chromosomal translocations. Sarcomas with complex karyotypes have high frequencies of p53 and Rb mutations as well as impairments in DNA repair and severe chromosomal instability. This group includes some of the more commonly diagnosed sarcomas; EWS, LMS, rhabdomyosarcoma (RD) and OSA. Many sarcomas that fall into both genetic subgroups also possess abnormalities in growth factor signaling and signal transduction pathways. Tyrosine kinases (TKs) make up the majority of defective signaling pathways in sarcomas, including mutations in the platelet-derived growth factor receptor (PDGFR), c-KIT, vascular endothelial growth factor (VEGF) and insulin-like growth factor-1
receptor (IGF1-R) signaling pathways. For example, GISTs, EWS, dermatofibrosarcoma protuberans (DFSP), synovial sarcoma and Kaposi’s sarcoma all have been shown to have mutations which elicit c-kit overexpression and/or PDGFR over-stimulation (1).

Tyrosine kinase inhibitors (TKIs) are a class of novel therapeutics that are effective alone and in combination with conventional chemotherapeutics in treating a variety of cancer subtypes. There are two basic classes of TKs, receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NRTKs). Upon TK activation, a cascade of protein interactions occur releasing signals of positive and negative regulators of a variety of cellular processes including cell cycle regulation, proliferation, adhesion, migration, invasion, transcription and survival. Under normal conditions, cellular signaling tightly regulates activated TKs. The induction of TK signaling in the oncogenic state overcomes controlled regulation and signaling becomes activated by a myriad of cellular mechanisms including mutation and over-expression of the TK receptors or receptor ligands. All TKs rely on adenosine triphosphate (ATP) to mediate the transfer of energy in the kinase domain and to elicit downstream signaling cascades via pathway intermediates. TKIs employ two strategies: (1) antibodies which act as receptor antagonists or to sequester the TK ligand, preventing the ligand from binding to the RTK, and (2) small molecule inhibitors which act by competing for the ATP-binding domain in the catalytic site of the enzyme (Figure 1).

The use of TKIs for the treatment of sarcomas is predicated on the hypothesis that malignant cells rely more heavily on TK signaling than neighboring normal cells. GIST tumors were previously untreatable with our current chemotherapeutic options. Advances in understanding the genetic nature of cancer have lead to the development of new
treatment for sarcoma. GISTs that harbor activating mutations in the \textit{c-kit} tyrosine kinase are sensitive to treatment with imatinib mesylate (Gleevec) as well as sunitinib malate, whereas those without \textit{c-kit} mutations are insensitive. Proof-of-principle of this concept has already been obtained in the sarcomas in the case of GISTs. TKIs currently under investigation as potential therapies for sarcomas are reviewed in Table 4.
Figure 1. Illustration of associated tyrosine kinases and targeted therapies. Several TKIs have been evaluated as potential therapeutics in sarcomas. TKI can inhibit TK signaling and activation by preventing ligand binding to receptors or by directly inhibiting the TK by binding in the catalytic domain of the kinase. Here, the targets of several TKIs are depicted.
Table 4. Development stage of TKI targeted agents in sarcoma

<table>
<thead>
<tr>
<th>Agent</th>
<th>Company</th>
<th>Targets</th>
<th>Stage of Development</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Small Molecule Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imatinib (Gleevec)</td>
<td>Novartis</td>
<td>c-KIT, PDGFR</td>
<td>Phase III GIST, Phase II DFSP, Kaposi’s sarcoma, Preclinical osteosarcoma, MFH,</td>
<td>(126, 127)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Ewing’s sarcoma</td>
<td></td>
</tr>
<tr>
<td>Gefitinib (Iressa)</td>
<td>AstraZeneca</td>
<td>EGFR family</td>
<td>Preclinical Ewing’s sarcoma, Preclinical osteosarcoma, rhabdomyosarcoma</td>
<td>(130)</td>
</tr>
<tr>
<td>Semaxanib</td>
<td>Pfizer</td>
<td>VEGFR, PDGFR</td>
<td>Phase II STS Preclinical neurogenic sarcomas, Ewing’s sarcoma</td>
<td>(132)</td>
</tr>
<tr>
<td>SU6668</td>
<td>Sugen</td>
<td>VEGFR</td>
<td>Preclinical Ewing’s sarcoma</td>
<td>(133)</td>
</tr>
<tr>
<td>CEP-5213</td>
<td>Cephalon</td>
<td>VEGFR</td>
<td>Preclinical angiosarcoma</td>
<td>(134)</td>
</tr>
<tr>
<td>CEP-7055</td>
<td>Cephalon</td>
<td>VEGFR</td>
<td>Preclinical angiosarcoma</td>
<td>(135)</td>
</tr>
<tr>
<td>NVP-AEW541</td>
<td>Novartis</td>
<td>IGF1-R</td>
<td>Preclinical osteosarcoma, Ewing’s sarcoma, rhabdomyosarcoma</td>
<td>(136)</td>
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<td>Dastinib</td>
<td>Brisol-Myers Squibb</td>
<td>Src</td>
<td>Phase II solid tumors</td>
<td>(137, 138)</td>
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<td>Sorafenib</td>
<td>Bayer</td>
<td>Ras</td>
<td>Phase II Kaposi’s sarcoma</td>
<td>(127, 136)</td>
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<tr>
<td>Perifosine</td>
<td>Keryx</td>
<td>Cell membrane signaling</td>
<td>Phase II STS</td>
<td>(139, 140)</td>
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<td>Sunitinib (Sutent)</td>
<td>Pfizer</td>
<td>Pan TKI, PDGFR, VEGFR</td>
<td>Phase III refractory GIST</td>
<td>(136, 141, 142)</td>
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<td><strong>Antibody Targeted</strong></td>
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<td></td>
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<tr>
<td>Herceptin (Tratusumab)</td>
<td>Genentech</td>
<td>HER-2/neu</td>
<td>Preclinical Ewing’s sarcoma</td>
<td>(132)</td>
</tr>
<tr>
<td>Bevacizumab (Avastin)</td>
<td>Genentech</td>
<td>VEGF</td>
<td>Phase II osteosarcoma, STS, Ewing’s sarcoma, Kaposi’s sarcoma, alveolar soft part sarcoma</td>
<td>(136, 141, 142)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Preclinical Ewing’s sarcoma</td>
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Applications of tyrosine kinase inhibitors in sarcomas

*c-KIT and PDGFR*

GISTs are one of the most common mesenchymal malignancies of the gastrointestinal system. Furthermore, advanced, unresectable or malignant GISTs are fatal and highly resistant to conventional chemotherapies (143, 144). Mutation in c-kit is a critical event in the development of this malignancy. It is estimated to occur in 75% to 92% of GISTs diagnosed (145, 146). Imatinib mesylate (Gleevec), a c-KIT, Bcr-ABL and PDGFR TKI, was originally utilized as a therapy for chronic myelogenous leukemia and is now used to treat GISTs. The first GIST patient received imatinib treatment in 2000. Within weeks this patient demonstrated a clinical response and remained stable for 18 months (122). Clinical trials were initiated in the United States and Europe following the dramatic results of this case-report. The first trials showed responses that ranged from 59 to 69%. However, approximately 12% of the patients on the initial trials experienced resistance to imatinib (147-150). Duensing et al. and Heinrich et. al. demonstrated that GISTs lacking c-kit mutations have a higher likelihood of demonstrating imatinib resistance, while tumors with c-kit mutations experienced at least a 50% reduction of tumor volume (149, 150).

A clinical trial consisting of 147 GIST patients randomly assigned to receive either 400 or 600 mg of imatinib daily, demonstrated that 53.7% of patients had a partial response, 27.9% had stable disease, and 0% achieved a complete response (151). A follow-up randomized clinical trial conducted by Verweij *et al.*, consisted of 946 patients randomly allocated 400 mg of imatinib either once or twice a day. Patients remained enrolled for an average of 760 days, and 56% of the patients that received imatinib once a
day progressed compared to the 50% of the patients who received the regiment twice-daily. The estimated hazard ratio for this study was 0.82 [95% CI 0.69-0.98]; p-0.026. There was a similar response with no significant difference between treatment arms: 5% of patients achieved a complete response, 47% achieved a partial response, and 32% achieved stable disease. This study concluded that the once daily 400 mg dose of imatinib is a sufficient treatment option for treating advanced or metastatic GISTs (152). Together, these studies provide the necessary evidence to recommend treating patients with advanced or metastatic GISTs containing c-kit and PDGFR mutations with imatinib for extended periods. There are numerous ongoing studies evaluating the role TKI; imatinib and sunitinib can be used in neoadjuvant, adjuvant, and metastatic settings alone or in combination with other agents.

DFSP is a slow growing sarcoma that is locally aggressive and has a high likelihood of local recurrence. Most DFSPs have a characteristic translocation, t(17;22). This places the regulation of PDGFβ, a ligand for PDGFR, under the control of the collagen 1A1 promoter, inducing overexpression of PDGFβ. In vivo experiments conducted in nude mice carrying tumors induced by DFSP-transformed cells treated with imatinib demonstrate significant inhibition of tumor growth (153).

A case-series of ten patients with advanced DFSP with the characteristic t(17;22) translocation were evaluated for clinical response to imatinib. Patients were treated with 400 mg of imatinib two times daily. Eight patients with local disease experienced a clinical response, four of which had a complete clinical response of an average duration of 220 days. The patients with metastatic disease had more complex karyotypes than those of the localized DFSPs. One patient with metastatic disease and the t(17;22)
translocation achieved a partial response (198 of 383 days of follow-up) but experienced disease progression seven months following treatment. The other patient with metastatic disease did not have the t(17;22) translocation and did not experience a clinical response. Based on this, imatinib is a viable option for the treatment of DFSP.

AIDS-related Kaposi’s sarcoma is associated with HIV and herpes virus/human herpes virus-8 co-infections usually found on the skin (126). Kaposi’s sarcoma has been shown to express high levels of both c-KIT and PDGFR, and imatinib has been evaluated as a potential therapeutic option for this sarcoma. In a study ten male patients with AIDS-related Kaposi’s sarcoma received 300 mg of imatinib twice daily for four weeks. Of the ten patients, five had partial clinical responses. The remaining five participants had stable disease at the end of the study, two of them with histological disease regression. This study demonstrates that imatinib has potential effectiveness as a treatment option for patients with AIDS-related Kaposi’s sarcoma (126).

TKIs inhibit proliferation over a range of sarcoma-derived cell lines in vitro and in vivo as measured by xenograft models in nude mice. Studies using imatinib have been completed in rat OSA and MFH cell lines expressing high levels of PDGFRα. Imatinib inhibited 20% and 40% of cellular proliferation, respectively when OSA and MFH cell lines were treated with 10 μM imatinib (154). OSAs have been shown to express high levels of PDGFR. However, preclinical studies have not shown imatinib to achieve anti-tumor activity within clinically relevant or achievable doses (155, 156).

The effects of imatinib were evaluated on a panel of eight different EWS cell lines and found that imatinib inhibited proliferation by 50% and induced apoptosis at IC_{50}s ranging from 10-12 μM. Furthermore, in xenograft models, imatinib treatment resulted
in regression and/or stabilization of primary Ewing’s tumors (157). Druker et al., also
determined a synergistic effect on growth inhibition when 10 μM imatinib was combined
with increasing doses of doxorubicin (134). Phase I clinical trials have demonstrated that
the maximally tolerated dose of imatinib is 1000 mM/day (148, 158, 159), which
corresponds to a concentration between 6-10 μM in the blood (160). This concentration
is below the IC50 required to inhibit the proliferation and induce apoptosis of EWS cell
lines (161). However, a study conducted on a panel of eight EWS cell lines with high
levels of c-KIT expression, found the cell lines to be resistant to imatinib at
concentrations ranging from 0.1 to 10 μM as determined by proliferation assays (162).
The preclinical data is conflicting in EWS and may be caused by inconsistencies among
the basic science experiments performed or may be inherent in the nature of the cell lines
utilized in these studies. Therefore, the preclinical data suggests that c-KIT is not a
critical target for EWS survival and thus may not serve a single agent treatment option.

EGFR/HER-2/neu

Gefitinib (Iressa), an epidermal growth factor receptor (EGFR) inhibitor, has
shown potential anti-tumor effects in several sarcoma cell lines when used in
combination with irinotencan, a topoisomerase I inhibitor (129). Murine xenograft
models have been used to assess the effects of gefitinib on implanted tumor activity.
Gefitinib was orally administered at 100 mg/kg either once or twice daily for 5
days/week. The anti-tumor activity of the combination was greater than additive in one
of the seven cell line-derived xenografts and enhanced the activity of irinotecan in three
of the seven xenografts. These data also reported an increased bioavailability of
irinotecan when used in combination with gefitinib (129).
A phase I trial of gefitinib was completed on 25 children with refractory solid tumors. Twelve of the 25 patients enrolled were sarcoma patients. Gefitinib was administered once daily for a course of 28 consecutive days starting at 150 mg/m² and escalated to 500 mg/m² a median of 54 courses were delivered. A recurrent EWS patient experienced a partial response after one course of treatment which lasted for ten weeks. The maximum tolerated dose in this study was 400 mg/m²/day and was tolerated well (128).

There is conflicting evidence in the literature evaluating HER-2/neu as an effective target in treating sarcoma, thus the potential role of HER-2/neu as a therapeutic target for sarcoma is controversial. One group evaluated five EWS cell lines and 13 archival primary EWS samples for HER-2/neu gene amplification and protein expression. While several of the EWS cell lines and tumor samples had high HER-2/neu protein expression, none had HER-2/neu gene amplification. In addition, upon assessment of trastuzumab, (Herceptin) an anti-HER-2/neu monoclonal antibody that inhibits HER-2/neu expression and blocks tumorigenesis induced by HER-2/neu, the group determined that trastuzumab had minimal inhibitory effects on cell growth, survival or colony formation of the EWS cell lines evaluated (140). Furthermore, others have evaluated trastuzumab alone and in combination with conventional chemotherapeutics used to treat sarcomas in two EWS cell lines. These studies have not been presented anti-tumor activity at clinically achievable doses (139) and it was concluded that HER-2/neu was not a major therapeutic target for the treatment of EWS. These results argue that HER-2/neu is not a critical pathway for the pathogenesis of EWS and targeting this pathway may have little therapeutic benefit in EWS patients.
**VEGFR**

Semaxanib is a dual VEGFR and PDFGR inhibitor that has been evaluated in neurogenic sarcoma cell lines and human tumor explants. Semaxanib has no effect on cell lines *in vitro* at doses up to 200 μM; however, it reduces proliferation in tumor explants by 54.8% in mice treated with 25 mg/kg/day for eight days. This growth reduction was due to decreased tumor angiogenesis, which led to decreased proliferation and increased apoptosis (131). These data suggest that VEGF may serve as a viable target in preventing angiogenesis in neurogenic sarcomas.

VEGFR inhibitors have been shown to be potent inhibitors of EWS growth in mouse models. Several inhibitors of VEGF signaling have been evaluated in EWS and have shown significant reduction of tumor growth in mouse models. The VEGFR inhibitors, SU6668 at 25 mg/kg/day and semaxanib at 100 mg/kg/day and anti-VEGF agents bevacizumab (avastin) at 10 mg/kg twice weekly and VEGF Trap at 2.5 or 25 mg/kg twice weekly significantly reduced tumor growth at clinically achievable doses (132). Another VEGF signaling inhibitor, CEP-5214, and its pro-drug CEP-7055 inhibit tumor growth in a mouse angiosarcoma model. CEP-5214 administration at 1-3 mg/kg/day for 10 days achieved the minimum effective dose and the maximum efficacy was observed by treating with 23.8 mg/kg CEP-7055 for 10 days (133). These preliminary data demonstrate the anti-tumor activity of these drugs and offer evidence to further investigate the possible therapeutic potential of these inhibitors for the treatment of angiosarcoma.

Therapeutics that inhibit TK signaling through the use of antibodies are currently undergoing clinical trials in sarcomas. Bevacizumab, a recombinant VEGF monoclonal
antibody, has shown clinical promise by significantly increasing disease-free progression and overall survival in several malignancies including breast, renal cell and colorectal cancers (136). A phase II trial combining doxorubicin at 75 mg/m² with 15mg/kg bevacizumab intravenously every three weeks was completed in patients with metastatic soft tissue sarcomas. A 12% response rate was observed in this study, and 65% of the patients experienced stable disease for four or more cycles (142).

A case-report of a nine-year-old boy demonstrated that bevacizumab has potential activity for treating alveolar soft part sarcoma. The patient was treated with bevacizumab intravenously for 1 hour at 5 mg/kg biweekly for four cycles, then increased to 10 mg/kg with no substantial side effects. He experienced a reduction of tumor load at all sites, including lung metastases and the patient was in good condition after 26 cycles of treatment (141). Additionally, there are several ongoing clinical trials investigating the use of bevacizumab as a possible therapy for sarcoma. The National Cancer Institute (NCI) is sponsoring clinical trials evaluating bevacizumab as a single agent and in combination with sorafenib (another TKI) in Kaposi’s sarcoma. In addition, there is an open-label phase II trial evaluating bevacizumab as a single agent in the treatment of angiosarcoma (136). Further investigation of bevacizumab in combination with conventional chemotherapies for the treatment of metastatic sarcoma is therefore warranted.
Other TKIs

Several additional TKIs are currently under investigation. IGF-1R is a target of great interest for the treatment of sarcomas. The therapeutic potential of NVP-AEW541, an IGF-1R inhibitor, was evaluated in a panel of 8 OSA, 10 EWS and 5 RD cell lines. NVP-AEW541 induced cell cycle arrest in a dose-dependent manner in the cell lines tested when treated with 300 nM, 1 μM and 3 μM for 24 hours. Apoptosis was induced in all of the EWS cell lines and in many of the OSA and RD cell lines, which correlated with IGF-1R inhibition. (134).

Perifosine targets cell membrane signaling pathways and has undergone phase I and phase II clinical trials in patients with advanced, metastatic solid tumors, including sarcomas. In the phase I study, 5 of 42 patients enrolled were sarcoma patients. One LMS patient was the only patient of the 42 total to experience a partial response. Six months following therapy, the patient reported elevated energy levels and symptom relief, and she experienced no significant disease progression once off all anticancer therapies (138). A phase II clinical trial consisting of 16 adult patients with locally advanced or metastatic STS were treated with perifosine. There were no clinical responses observed in this study. Four patients had stable disease which lasted 1.3 to 8.2 months (137). The preliminary clinical data may have suggested potential therapeutic benefit from treatment with perifosine; however, the phase II trial does not provide compelling evidence to suggest a high degree of anti-tumor activity.

The Raf kinase inhibitor sorafenib is currently under investigation in National Cancer Institutes sponsored studies for the treatment of metastatic and locally advanced sarcoma (136). Sunitinib (Sutent), a pan-TKI that elicits inhibitory effects on a variety
of TKs including PDGFR α and β and VEGFR 1-3 has recently been approved for refractory GIST patients (136). A double-blind, phase III trial conducted to determine the efficacy of sunitinib in patients with GISTs that failed imatinib treatment demonstrated that patients had a significant increased progression free duration of 6.3 months compared to 1.5 months in the placebo controls (127). Semaxanib, a reversible VEGFR2 and PDGFR inhibitor, underwent phase II clinical trials investigating its use in patients with relapsed soft tissue sarcoma. Anti-tumor activity was encouraging with 23% observed responses when treated with 145 mg/m² twice weekly (130).

There is considerable evidence to suggest that TKIs may serve as effective treatments options for sarcoma either alone or in combination with other chemotherapies. A protein kinase that serves as a point of convergence for many of these targeted TK signaling pathways is Src kinase. Our preliminary data has shown that Src is constitutively activated in sarcoma tissues. Therefore, Src may be a worthwhile target for treating sarcomas.
History of Src: 1911- present

Nearly a century Peyton Rous discovered a “filterable agent”, or virus, that caused sarcoma in chickens (163, 164). This virus, later called Rous sarcoma virus (RSV), has remained in mainstream cancer research ever since its identification. However, it was some 30 to 40 years after its initial discovery that the scientific community would come to accept that idea that a virus could contain genetic information which induced tumors. The road to establishing the foundation of identifying v-Src, the oncogene that causes the RSV has been long. In 1941 it was shown that chicken embryo fibroblasts growing in culture infected with RSV adopted round and spindle-shaped morphology, similar in characteristic to Rous sarcoma cells (165). The focus assay was developed to quantify the number of cells infected with RSV in the early 1950s. By the late 1950s, RSV was shown to induce tumors in mammals by many groups (166-170). These findings later, proved significant in identifying the transforming ability of Src protein. The transformation abilities of RSV mutants were identified in the 1960s, in which mutations induced spindle-shaped cells, rather than rounded cells (171). This discovery, among others, lead to the 1970 identification of the oncogene responsible for cellular transformation of RSV, v-src. Then in 1977, a 60 kDa phosphoprotein immunoprecipitated from RSV tumor-bearing rabbits was identified as the src gene product (172). A year later two independent groups working on Src demonstrated that Src was a protein kinase (173, 174) and more specifically, a tyrosine kinase in 1979 by Tony Hunter (175).

The discovery that RSV required a specific gene for its transforming capability shed light on a new way of studying the role genes play in cancer. The idea of the
existence of “oncogenes”, transforming genes found in RNA tumor viruses, lead to the idea that proto-oncogenes may exist as normal cellular counterparts. Proto-oncogenes are precursors of viral transforming genes, which they themselves lack the ability to transformation cells unless mutated or overexpressed. The first of these to be identified was c-src (176, 177), the discovery of which initiated a fury of research into the role of proto-oncogenes in cancer.

Upon sequencing c-src, it was revealed that the cellular and viral versions differ in that v-src has carboxy-terminal substitutions and deletions, which were later linked to the aberrant activity of v-Src protein (176). Upon cloning of the c-src gene lead to comparing the biological activity of both forms of the gene. Except when overexpressed, c-Src has lower transforming activity in mammalian and avian fibroblast cell lines (178-180). Determining how the transforming ability of c-Src was activated lead to characterization of the c-src protein product. The human Src gene encodes a 536 amino acid protein, three more amino acids than the chicken ortholog.

It is now known that Src is one of 11 members of the human Src-family Kinases (SFK), which also includes Blk, Brk, Frk, Fyn, Hck, Lck, Lyn, Srm and Yes (124). Fyn, Src and Yes are expressed in all cell types, Srm is found in keratinocytes and Blk, Fgr, Hck, Lck and Lyn are primarily found in hematopoietic cells. While Frk is expressed in bladder, brain, breast colon and lymphoid cells, and Brk is expressed in colon prostate and the small intestine (181).

SFKs share similar homology and protein organization. The organization of Src protein from N- to C- termini contains a 14-carbon myristoyl group, which facilitates the attachment of Src to membranes and is required for normal function, is attached to an
SH4 (Src Homology) domain. This unique domain is followed by an SH3 and SH2 domain, an SH2 kinase linker, a protein kinase domain containing the active site, and the C-terminal regulatory domain (Fig. 2). The SH2 and SH3 domains have several important functions, including constraint of enzyme activity via intramolecular contacts during periods of inactivity and binding to ligands with SH2 and SH3 domains to attract them to specific locations within the cell. There are two vital tyrosine phosphorylation sites on Src, Tyr530 and Tyr419. When phosphorylated, Tyr530 binds intramolecularly to the SH2 domain, stabilizing the restrained enzyme and preventing interaction of SH2 and SH3 with other ligands (Fig. 3). In normal cells Src activity is tightly regulated, 90-95% of Src is phosphorylated on Try530 and in an inactive state and becomes transiently activated during specific cellular event such as mitosis (182). Csk and Csk homologous kinase (Chk) are two cytosolic tyrosine kinases responsible for phosphorylation of Tyr530. Tyr419 is an autophosphorylation site that is located in the active site of the enzyme and promotes Src kinase activity. Phosphorylation of Try419 stabilizes the active confirmation. Thus Tyr419 is the activation phosphorylation site and Tyr530 is the inhibitory phosphorylation site of Src which is missing from the v-Src protein (183). When phosphorylated on Tyr530, Src cannot undergo autophosphorylation, Tyr530 must first be dephosphorylated. However, when phosphorylated on Tyr419 and Tyr530 simultaneously, the Tyr419 phosphorylation overrides the inhibitory Tyr530 and remains active (184).

Src activation is initiated by several cellular mechanisms including receptor tyrosine kinases, integrin receptors, cytokine receptors, G-protein receptors and steroid hormone receptors (185). Once activated, Src signals to the nucleus through a variety of
downstream effectors including STAT3 (186, 187), PI3K (188, 189) and Ras (190). Src plays a role in a myriad of cellular functions including proliferation, survival, cell adhesion, migration, cellular morphology and bone resorption. For example, under normal conditions, when epidermal growth factor receptor (EGFR) is activated, Src becomes activated during G2/M transition through the cell cycle. Src activity has also been shown to be an important characteristic of osteoporosis. Mice with Src -/- null mutations die within weeks of birth and express an osteoporosis phenotype, resulting from increased bone resorption caused by a defect in osteoclasts (191). Thus, Src kinase inhibitors currently serve as targeted therapy option for treating osteoporosis.

**Figure 2.** Organization of Src kinase. Structural features of c-Src compared to v-Src protein. Both proteins are myristoylated at Gly2 contain membrane binding (MB), unique (U), SH3, SH2, linker and catalytic domains. Notice only c-Src possesses the inhibitory Tyr530, or regulatory (Reg) domain at the C-termini. c-Src is usually phosphorylated on Tyr530 and in the inhibitory conformation, while v-Src is phosphorylated at Tyr419 and constitutively active because the regulatory domain is missing. Other differences between the two proteins include amino acid substitutions in v-Src.
Figure 3. Src activation and signaling conformations. The left panel represents the inactive conformation of the c-Src protein. When phosphorylated, Tyr530 binds to the SH2 domain and prevents phosphorylation of the active site, Tyr419, while the SH3 domain interacts with the linker domain, further stabilizing the conformation. Upon activation of the active site the SH2 domain releases Tyr530, SH3 releases the linker domain and they are now available to interact with other proteins containing SH domains. Furthermore, the active site is open or active, available to elicit signals (right side of panel).
Considerable evidence suggests that Src may serve as an option for targeted therapeutics in cancer as well. Src kinase activity has been shown to be constitutively activated in several types of human cancers including breast, prostate, colon, pancreatic and just recently sarcoma as compared to normal tissues. For example, Src is overexpressed in ~70% of breast cancers (192). Furthermore, the increased activity of Src is strongly implicated in the development, growth, progression and metastasis of cancer. Mutations in the c-Src gene are very rare, therefore overexpression may be induced by increased activation and association with RTKs. Reduced levels of Csk or Chk activity and decreased phosphatase activity within the cell may also be potential mechanisms for increased Src activity in cancer (193).

The increased activity of Src in cancer induces dramatic alterations in signal transduction of downstream transcriptional events. Many of these downstream targets have been linked to tumorigenicity and metastasis (194). One widely studied pathway involved in tumorigenesis and downstream of Src activation is STAT3, a member of the signal transducers and activators of transcription (STAT) family. STATs are mediators of Src induced cellular transformation. This model of evidence introduced in 1995, showed that the DNA-binding activity of STATs was enhanced in cells transformed with v-Src (187). Upon activation, Src phosphorylates STAT3, which homodimerizes and translocates to the nucleus where it regulates the transcription of genes involved in cellular proliferation, survival and migration. It is not surprising considering that STAT3 is activated by Src to learn that STAT3, along with other STAT proteins, are constitutively activated in diverse human cancer cell lines and tissues. In cancer, STAT3
regulates the transcription of genes involved in uncontrolled tumor cell proliferation, resistance of apoptosis, evasion of the immune system and angiogenesis, among other malignant phenotypes (195). Consequently, STAT proteins and their activators have emerged as potential targets for cancer therapy (196, 197).

In addition to STATs, increased Src activity has been associated with adhesion changes characteristic of mesenchymal transition and is suggested to promote cancer cell migration and metastasis. Focal adhesion kinase (FAK), along with Crk-associated substrate (CAS) are substrates of Src and serve as focal adhesion proteins vital for integrin signaling. FAK, also a tyrosine kinase, modulates the formation and turnover of focal adhesions, the intracellular structures that link the extracellular matrix to the actin cytoskeleton (198). Src and FAK associate upon autophosphorylation of the Tyr397 residue on FAK, after which Src becomes autophosphorylated and can then phosphorylate several residues on FAK, including Tyr576/577 and Tyr925. Phosphorylation of FAK by Src enhances FAK activity.

Mutational studies have shown that the inactivation of FAK is embryonic lethal (199) and selective inhibition of fak expression in skin keratinocytes suppressed chemically induced tumor formation in vivo (200). Others have shown that inhibition of fak expression induced apoptosis in vivo and in vitro in keratinocytes (200) and endothelial cells in vitro (201). Furthermore, the interplay between Src and FAK is important for modulating the gene expression of transcription factors and cell motility proteins, as elucidated in deletion and rescue experiments (202). Overexpression of FAK suppresses p53-mediated apoptosis by binding to the transactivation domain of p53 and limiting transcription activity (203). While, FAK itself does not function as an oncogene,
elevated expression in cancer correlates with increased cell motility, invasiveness and proliferation (202, 204). RNAi-mediated knockdown of FAK in an aggressive breast carcinoma cell line did not affect cellular proliferation, but cells lacking FAK lacked invasive characteristics \textit{in vitro} and lacked spontaneous lung metastasis \textit{in vivo} (205). In addition, FAK expression, along with Src, increases with colon cancer invasiveness (206).

The FAK/Src complex also phosphorylates CAS, a cytoskeletal adapter protein. Upon Src-mediated phosphorylation, CAS acts as docking protein for multiple protein–protein interaction domains and is important for the recruitment of adapter proteins, including Crk, to the substrate domain of CAS (207). CAS activity has been shown to promote the invasiveness of Src-transformed cells (208) and play a vital role in cell motility and survival. While the precise mechanism of CAS mediated apoptosis is unknown, phosphorylation of CAS and its association with FAK have been shown to be essential in the survival pathway. Furthermore, expression of antisense CAS partially reversed the transformation activity of v-Src, suggesting that the inhibition of CAS plays a direct role in cellular transformation (209).
Development of Src inhibitors for clinical trials

Models have demonstrated that overexpression and activation of Src is associated with many oncogenic characteristics such as disruption of the cell cycle, increased migration and invasion and protection from apoptotic stimuli (193). Given the history of Src and because it plays such a pivotal role in many oncogenic processes, it is surprising that Src has only recently been widely considered as a potential therapeutic target for cancer therapy. There are three variety of Src inhibitors; SH2/SH3 blocking inhibitors, Src destabilizing agents and ATP-competitive inhibitions. SH2/SH3 blocking inhibitors prevent the SH domain mediated interactions and prevents Src protein-protein interactions. These inhibitors have demonstrated poor transport and uptake properties and only inhibit a subset of Src protein interactions, limiting the clinically efficacy of this class of Src inhibitors (210-212). Src destabilizing agents interfere with the association of Src and its molecular chaperone Heat Shock Protein (HSP) 90. These agents have proven to be non-specific and results in the disruption of many HSP interactions, which could result in multiple adverse side-effects in patients (213, 214). ATP competitive Src kinase inhibitors target the active site and prevent ATP from binding and initiating the phosphotransferase activity of the enzyme. There is significant homology in the structure and sequence of ATP-binding domains of kinases, which has made the narrowing the specificity of ATP competitors difficult.

However, several ATP competitive inhibitors are currently under various stages of investigation as potential therapies for cancer. PP1 and PP2 were among the first Src kinase inhibitors utilized to study the role of Src activity in cellular events. The use of PP2 has shown that inhibition of Src results in a loss of downstream Src signaling.
through pathways including FAK, Akt and STAT3 and elicits anti-tumorigenic phenotypes (193). These findings were later proven to be through Src inhibition using siRNA to knockdown Src expression (215). Since the discovery that inhibition of Src can lead to anti-tumorigenic effects in cancer models several ATP-competitive Src kinase inhibitors have been synthesized and studied. Three such inhibitors include PD180970 (Pyridol[2,3-d] pyrimidine), SKI-606 (4-anilino-3-quinolinecarbonitrile) and dasatinib (BMS-354825, [N-(2-chloro-6-methylphenyl)-2-(6-(4-(2-hydroxyethyl) piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide) (Fig. 4), all of which have been thoroughly studied in epithelial and hematopoietic cancers.

PD180970 was the first of the three to undergo investigation as a Src kinase inhibitor. Several groups have shown that PD180970 can inhibit Src at nanomolar doses (216-219). Most significantly, others have shown that the inhibition of Src-STAT3 signaling in cancer cells can lead to the induction of apoptosis (216, 219). However it is a non-specific kinase inhibitor, only soluble in DMSO and is highly unstable, which leaves this compound with little to no clinical future. SKI-606 is an orally available compound with dual specificity for SFKs and Abl tyrosine kinase (220). Studies have shown that SKI-606 decreases growth and motility of colorectal cancer cells by preventing activation of Src and downstream signaling (221). Furthermore, SKI-606 has been shown to have anti-proliferative effects in colon cancer and myelogenous leukemia cancer models (220, 222). These data have lead to the initiation of clinical trials in patients with hematopoietic and epithelial cancers. Dasatinib, is also an orally available Src inhibitor that is currently undergoing clinical trial in epithelial cancers. Preclinical data have shown dasatinib to be a potent inhibitor of Src activity in prostate, head and
Figure 4. Structure of three ATP-competitive Src kinase inhibitors. A, PD180970, B, SKI-606 and C, dasatinib were independently developed and provided by three different pharmaceutical companies. All three inhibitors act as ATP-competitors for the catalytic domain of Src kinase and prevent the activation of Src by phosphorylation of Tyr419.
neck and lung cancers (223-225). Nam et al., showed dasatinib inhibition of Src at nanomolar concentrations inhibited downstream signaling of STAT3 and FAK which subsequently inhibited the migration and invasive properties of prostate cells (223). While others have shown that in addition to inhibiting migration and invasion, dasatinib also induces cell cycle arrest and apoptosis in head and neck (224) and lung cancer cell lines (225).

There is remarkable evidence to suggest that Src kinase activity may serve as an important target for the treatment of cancer. Until the culmination of this dissertation research, the role of Src activation in human sarcoma had not yet been elucidated. In addition, the responses and mechanism of action of ATP-competitive Src kinase inhibitors in mesenchymally-derived tumor cell lines have not been described previously. There is considerable evidence to suggest a role for Src in sarcomagenesis. Vigneron et al., have shown that increased Src expression in sarcoma cell lines reduced sensitivity to conventional chemotherapies used to treat sarcomas. This chemo-resistance was associated with a failure of cells to up-regulate p21 in response to adriamycin (226). Furthermore, increased Src activity has also been shown to contribute to anoikis resistance in human OSA cell lines. Inhibition of Src activity restored anoikis sensitivity and cells underwent apoptosis upon detachment (227). Thus, these three independently synthesized compounds were evaluated in sarcoma cell lines to identify the role of Src kinase activation in the sarcoma cell lines. The objective of this dissertation was to determine the role of Src activation in sarcomas and determine whether Src may serve as a viable option for the treatment of sarcoma patients.
The overall objective of the studies conducted for this dissertation was to investigate the role of Src kinase activation in the survival and malignant phenotype of sarcomas. Src kinase was the original oncogene identified in Rous Sarcoma Virus (172). Previous research has suggested a possible role for Src activation in tumor survival, in that Src is involved in a myriad of cellular mechanisms such as survival, migration and invasion. However, the role of Src in human sarcomas had not been determined. Results from preliminary experiments suggested that Src kinase is activated in a variety of human sarcomas. The recent development and interest of Src kinase inhibitors aided in evaluating Src as a possible target of sarcoma treatment. These findings in addition to the overwhelming evidence suggesting that Src may serve a possible target essential for the survival of sarcomas lead to the development of the hypothesis that Src is required for the migration, invasion and survival of sarcomas. To verify this hypothesis, the following aims were pursued.

**Aim 1: Evaluate the biological response of three Src kinase inhibitors in sarcoma cell lines.**

A. **To determine the role of Src kinase activation in sarcomas cells lines.** Src kinase has been shown to be activated to varying degrees in primary sarcomas. However, the degree of activation and significance of Src activation in sarcoma
The role of Src kinase activity in sarcoma cell lines has not been determined. Because Src was originally identified as an oncogene significant in the development of sarcoma in chickens, we sought to determine the role of Src kinase activity in sarcoma cell lines using three different Src kinase inhibitors.

B. To determine if STAT3 activation is required Src activated migration, invasion and survival of sarcomas. STAT3 is among multiple downstream signals initiated by Src activation. STAT3 was evaluated as a possible signaling protein involved in the cellular events elicited by Src activation in sarcoma cell lines.

Aim 2: Identify a molecular signature that predicts response to dasatinib by induction of apoptosis in sarcoma cell lines.

A. Identify a gene expression profile unique to sarcoma cell lines that undergo apoptosis when treated with dasatinib. Src kinase has previously been shown to be essential for tumor survival in epithelial derived tumors (185, 207, 228-230). The data from aim 1 has shown that Src kinase activity is required for survival in a subset of sarcoma cell lines. Therefore, microarray analysis was performed on sarcoma cell lines to identify a molecular signature that predicts response to dasatinib by induction of apoptosis in sarcoma cells.

B. Confirm the predictability of the molecular signature to predict response to dasatinib in an independent dataset. It is hypothesized that a molecular signature may be identified in tumors and tumor cell lines which may predict the response to specific therapies. To test the effectiveness of the molecular
signature that predicts response to dasatinib two new cell lines of unknown response status were utilized. The cell lines were used to determine whether the molecular signature extracted from the responsive cell lines was successful in identifying new cell lines that would or would not respond to dasatinib by induction of apoptosis.

Aim 3: Verify the presence of the molecular signature in primary human sarcoma specimens.

A. Determine if the molecular signature identified in the sarcoma cell lines is present in untreated, primary human sarcoma specimens. Cell lines lack the microenvironment interactions of human tumors and one could not be certain that the molecular signature identified in the cell lines is expressed in human tumors. Untreated sarcoma specimens were utilized to establish whether the molecular signature unique to the cell lines that respond to dasatinib was present in human tumors. Microarray analysis was performed on a diverse set of human sarcomas to validate the expression of the cell line signature in tumors.

B. Establish whether the molecular signature can cluster sarcomas based on expression and theoretically predict response to dasatinib. Once the molecular signature was identified in primary sarcomas, the signature was used to predict theoretical response to dasatinib. The molecular signature was further tested to determine if tumors clustered into the respective theoretical categories based on the expression of the molecular signature.
Materials and Methods

Cells and reagents

SaOS-2, U 2 OS, MG-63, SK-ES-1, A673, RD, SK-LMS-1, HT-1080, SW-872, HOS and SW1353 sarcoma cell lines were obtained from the American Type Culture Collection. The LM2 and LM7 OSA cell lines were provided by Dr. Eugenie S. Kleinerman of MD Anderson Cancer Center (Houston, TX). The TC-71 EWS cell line was provided by Dr. Timothy Triche of the University of Southern California (Los Angeles, CA). The SaOS-2, MG-63, LM2, LM7, TC-71, SK-LMS-1, HT-1080 and SW-872 cell lines were maintained in MEM supplemented with Eagle’s salts, 10% fetal bovine serum, 2-fold MEM vitamins, 1 mM sodium pyruvate, 1 mM non-essential amino acids and 2 mM L-glutamine. The SK-ES-1 and U 2 OS cell lines were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum. The A673 cell line was maintained in DMEM supplemented with 10% fetal bovine serum. The RD and RD18 cell lines were maintained in DMEM/F12 (1:1) supplemented with 10% fetal bovine serum. All cells were maintained at 37°C in 5% CO2. All experiments were performed using exponentially proliferating cells unless otherwise noted.

Polyclonal antibodies to phosphorylated p-Src (Y419), p-FAK (Y576/577), p-p130CAS (Y410), pSTAT3 (Y705) proteins, and to total FAK and PARP proteins were obtained from Cell Signaling Technologies (Cambridge, MA). Polyclonal antibodies to p130CAS and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to total Src (clone GD11) and hILP/XIAP were obtained from
Upstate Biotechnology (Lake Placid, NY) and BD Biosciences (San Diego, CA), respectively. Dasatinib was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ). Dasatinib was synthesized by the addition of methylprimididine to the 2-amino group of thiazole, followed by a reaction with hydroxyethyl piperazine (231).

Preparation of cell extracts and Western blotting

Adherent sarcoma cells were washed with ice cold 1x PBS, followed by washing and scraping from plates in ice cold 1x PBS containing 5 mM sodium fluoride and 1 mM sodium orthovanadate (1x PBS + inhibitors). Cells were harvested by centrifugation, washed with 1x PBS + inhibitors and harvested again by centrifugation. Pellets were resuspended in RIPA buffer containing 0.1 mM Na₃VO₄, NaF, DTT and 1:100 fold dilution of protease inhibitor cocktail obtained from Sigma (St. Louis, MO). Samples were vortexted for 30 min at 4°C and insoluble material was removed by centrifugation. For Western blotting, 50 μg of protein was resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked in 1x TBS containing 0.01% Tween-20 and 5% non-fat dry milk for 30 min, and then incubated with primary antibody in 1x TBS containing 0.01% Tween-20 and 5% BSA obtained from Sigma (St. Louis, MO) overnight at 4°C with rocking. Protein-bound primary antibodies were detected using respective horseradish peroxidase-coupled secondary antibodies (Amersham anti-rabbit for polyclonal and anti-mouse for monoclonal, obtained from GE Healthcare Unlimited, Buckinghamshire, UK) diluted 1:10,000 in 1x TBS containing 0.01% Tween-20 and 5% non-fat dry milk and incubated for 2 h at room temperature. Bound secondary antibodies
were detected using Amersham ECL PLUS Western blotting detection reagents obtained from GE Healthcare Unlimited. Densitometry was performed on p-Src (Y419) Western blots performed for dose responses in each of the cell lines and then analyzed using ImageQuant 5.2 (Molecular Dynamics) software. Percent inhibition of Src kinase activity as measured by Src (Y419) phosphorylation was determined by nonlinear regression analyses and data were reported as the inhibitory concentration required to achieve 50% inhibition relative to control reactions (IC$_{50}$) in Table 1. Data are the averages of triplicate determinations.

**Immunohistochemistry**

Human tissues were obtained through the Moffitt Cancer Center Tumor Bank using IRB-approved protocols. Tissues were fixed in formalin within 15-20 min from the moment of surgical excision to preserve the phosphorylation status of proteins such as Src and STAT3. Formalin-fixed, paraffin-embedded tissue sections of 3 μm thickness were deparaffinized by an initial warming to 60°C, followed by two xylene changes of 10 min each, two series of 30 dips in absolute alcohol, 30 dips in 95% alcohol, and 20 dips in deionized water. Slides were placed for 5 min in TBS/Tween and processed on a DAKO Autostainer using the Dako LSAB+ peroxidase detection kit (Carpinteria, CA). Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide followed by 20 dips in deionized water. The anti-p-Src (Y419) or pSTAT3 (Y705) was applied at 1:100 dilution for 30 min after microwave antigen retrieval with 0.1 mol/L citrate buffer (pH 6.0; Emerson 1,100 W microwave, high to boiling, then 20 min on power level 5). The chromogen 3,3’-diaminobenzidine was used for detection. Counterstain was done with
modified Mayer's hematoxylin. Slides were dehydrated through graded alcohol, cleared with xylene, and mounted with resinous mounting medium.

**Src Family Kinase PCR**

A multiGene-12 RT-PCR Profiling Kit was obtained from SuperArray Bioscience Corporation to determine the status of gene expression for 11 SFK members in eight sarcoma cell lines; SaOS-2, LM-2, LM-7, U2 OS, MG-63, SK-LMS-1, HT1080 and SW872 cell lines. RNA was isolated from exponentially growing cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. First strand cDNA synthesis was completed using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The remainder of the RT-PCR protocol was completed according to the SuperArray protocol. Once resolved in a 1% agarose gel, photographs were taken for each of the cell lines to compare gene expression.

**Wound healing assay for cell migration**

Cells were plated in 24 well tissue culture plates, grown to confluency and serum starved in 0.1% FBS overnight. Monolayer wounds were produced using a pipette tip scratched through the center of the well. Photomicrographs were taken of the initial wound for comparison. Cells were then treated with either DMSO alone, as vehicle control or escalating doses of dasatinib and allowed to migrate into the denuded areas for 24 h. Following incubation, cells were briefly stained with Coomassie blue. Cell migration was visualized at 10x magnification and digitally photographed. The distance
of migration was measured as pixel units and compared to time zero. The average
number of pixel units measured in the denuded area were determined from wound
healing assays for dose responses in each of the cell lines analyzed. Percent inhibition of
migration was determined by nonlinear regression analyses and data were reported as the
inhibitory concentration required to achieve 50% inhibition relative to control reactions
\((IC_{50})\). Data are the averages of triplicate determinations.

**Cell invasion assay**

Cell invasion assays were performed following the BioCoat Matrigel Invasion
Chambers protocol, obtained from BD Biosciences (Bedford, MA). Briefly, cells were
tryptsinized and washed once with 1x PBS and twice using serum-free medium. Cell
suspensions were prepared at \(5 \times 10^4\) cells/mL in 0.5 mL containing DMSO alone as
vehicle control or escalating doses of dasatinib in serum-free medium and added to the
chamber insert. Chambers were incubated for 22 h at 37 °C and 5% CO₂. Following
incubation, non-invading cells were removed using a cotton-tipped swab. The cells that
invaded to the lower surface of the membrane were stained using a Diff Quick Staining
Kit from Fisher Scientific (Pittsburgh, PA), digitally photographed and counted. Each
experiment was completed in triplicate.

**TUNEL assay**

Apoptosis was detected by the TUNEL assay using the In Situ Cell Death
Detection Kit obtained from Roche Molecular Biochemicals (Indianapolis, IN) according
to the manufacture’s protocol. Cells were treated with either DMSO alone as vehicle control or escalating doses of dasatinib for 48 h.

**siRNA transfections**

siRNA directed specifically against c-Src and a non-targeting siRNA control were obtained from Dharmacon RNA Technologies (Chicago, IL). Cells were plated on 6 cm tissue culture plates in complete media \((5 \times 10^5\) cells per plate) and allowed to attach overnight. siRNA was transfected in escalating doses \((50\, \text{nM and} \ 100\, \text{nM})\) using Oligofectamine obtained from Sigma-Aldrich (St. Louis, MO). The transfection incubation time for the siRNA/Oligofectamine complexes was 24 h, and total incubation time before harvesting cell lysates was 72 h.

**Statistical analysis**

Descriptive statistics, such as mean values and standard deviation, were calculated for the biological effects of dasatinib on invasion by dose levels \((\text{nM})\). To determine statistical significance between pair-wise dose levels, the exact Wilcoxon two-sample test or T-test were used, considering the small sample sizes. One-sided tests at a significance level of 0.05 were examined. All data were analyzed using the SAS software (version 9.1, SAS Institute, Cary, NC).

**Microarray sample preparation**

Three consecutive passages of untreated, exponentially growing SaOS-2, LM2, LM7, U 2 OS, MG-63, SK-ES-1, SK-LMS-1, HT1080, A673, RD, RD|18, HOS and
SW1353 were utilized for microarray analysis. Sarcoma tissue was collected from the H. Lee Moffitt Tumor Bank. Tissues were untreated primary malignancies that were snap-frozen in liquid nitrogen within 15 minutes following removal from the patient to minimize cellular degradation.

**Isolation of RNA**

Total RNA was excised from sarcoma tissue specimens and cell lines using the TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The aqueous phase containing the RNA separated from the TRIzol reagent was further purified using the RNeasy clean-up procedure (Qiagen Inc., Valencia, CA). The quality of total RNA was assessed by agarose gel electrophoresis and the total RNA from the tissues was further analyzed on a Agilent 2100 Bioanalyzer.

**Preparation of labeled RNA targets for hybridization**

Within the total RNA pool the poly(A) RNA was specifically converted to cDNA and then amplified and labeled with biotin following the procedure initially described by Van Gelder et al. (232). First-strand cDNA synthesis was carried out using the Superscript Choice System (Invitrogen, Carlsbad, CA) and the T7 promoter/oligo (dT) primer (5’-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24-3’), (Genset Corp., La Jolla, CA). Following annealing the rest of the cDNA synthesis reaction was prepared such that the final reaction contains 5 μg RNA, 100 pmol T7-(T)24 primer, 500 μM each dNTP, 10 mM DTT, 50 mM Tris- HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, and 200 U of Superscript II reverse transcriptase (Invitrogen Corporation,
Carlsbad, CA). The reaction was incubated for 1 hr at 42°C. A second-strand cDNA synthesis was performed at 16°C for 2 hr in a total volume of 150 μL, using 10U of *E.coli* DNA ligase, 40 U of *E. coli* DNA polymerase I, and 2 U of *E. coli* RNase H in the presence of 200 μM of each dNTP, 10 mM (NH₄)SO₄, 1.3 mM DTT, 26.7 mM Tris-HCl, pH 7.0, 100 mM KCl, 5 mM MgCl₂, and 150 μM β-NAD⁺ (Invitrogen). Following the second-strand DNA synthesis, 10 U of T4 DNA Polymerase (Invitrogen) was added and the samples incubated an additional 5 min at 16°C. The reaction was stopped by the addition of 0.5 M EDTA and subsequently extracted with an equal volume of phenol/chloroform/isoamyl alcohol. The double-stranded DNA (dsDNA) will then be precipitated with the addition of 0.5 volumes of 7.5 M NH₄ Acetate and 2.5 volumes of ice-cold 100% ethanol. The dsDNA then serves as a template for a transcription reaction performed with the GeneChip IVT Labeling kit according to manufacturer's instructions (Affymetrix Corp., Santa Clara, CA) which incorporates biotinylated UTP into the transcripts. The Biotin-labeled RNA was purified using RNeasy columns (Qiagen) and fragmented to a size of 35 to 200 bases by incubating at 94°C for 35 minutes in fragmentation buffer (40 mM Tris-acetate, pH 8.1/100 mM potassium acetate/30 mM magnesium acetate). The integrity of the starting material and the products of each reaction were monitored on agarose gels to assess the size distribution of the products and compare them to the starting material.
Array hybridization and scanning

The hybridization solution consisted of 20 µg of fragmented RNA and 0.1 mg/ml sonicated herring sperm DNA, in 1x MES buffer (containing 100 mM MES, 1 M Na\(^{+}\), 20 mM EDTA, and 0.01% Tween 20). In addition the hybridization solutions were spiked with known concentrations of RNA from the bacterial genes, BioB, BioC, and BioD, and one phage gene, Cre, as hybridization standards. The hybridization mixtures were heated to 99°C for 5 min followed by incubation at 45°C for 5 min before injection of the sample into a probe array cartridge. All hybridizations were carried out at 45°C for 16–17 h with mixing on a rotisserie at 60 rpm. Following hybridization, the solutions were removed and the arrays were rinsed with 1x MES. Subsequent washing and staining of the arrays was carried out using the GeneChip Fluidics station protocol EukGE_WS2, which consists of 10 cycles of 2 mixes per cycle with non-stringent wash buffer (6x SSPE, 0.01% Tween 20) at 25°C followed by 4 cycles of 15 mixes per cycle with stringent wash buffer (100 mM MES, 0.1 M Na\(^{+}\), and 0.01% Tween 20) at 50°C. The probe arrays were then stained for 10 min in streptavidin-phycoerythrin solution (SAPE) [1x MES solution, 10 µg/ml SAPE (Molecular Probes, Eugene, OR), and 2 µg/µl acetylated BSA (Invitrogen)] at 25°C. The post-stain wash was 10 cycles of 4 mixes per cycle at 25°C. The probe arrays were treated for 10 min with an antibody solution [1x MES solution, 2 µg/µl acetylated BSA, 0.1 µg/µl normal goat IgG (Sigma Chemical, St. Louis, MO), 3 µg/µl biotinylated goat-anti-streptavidin antibody, (Vector Laboratories, Burlingame, CA)] at 25°C. The final wash consisted of 15 cycles of 4 mixes per cycle at 30°C. Following washing and staining, probe arrays were scanned once at 1.5-µm resolution using the Affymetrix GeneChip Scanner 3000.
Data analysis

Scanned output files were visually inspected for hybridization artifacts and then analyzed by using Affymetrix Micro Array Suite (MAS) 5.0 software. Arrays were scaled to an average intensity of 500 and analyzed independently. The MAS 5.0 software uses a statistical algorithm to determine the signal intensity of a transcript from the behavior of 11 different oligonucleotide probes designed to detect the same gene (233). Probe sets that yield a change p-value less than 0.05 were identified as changed. Gene changes were selected using one of two methods the Significance Analysis of Microarrays (SAM) technique of Tusher et al. (234) or by filtering the probe sets to contain only probe sets with a maximum/minimum intensity value ratio of less then 2.0 for responders and non-responders. Probe sets that fit both criteria were further examined for use in the molecular signature.

To test the reliability of the signature and determine the response status of two cell lines with unknown response to dasatinib the fold change of the probe sets that comprise the molecular signature was determined as compared to either the median intensity for each probe set for all samples as the comparison group. A decision on response was determined based on how the fold change compared to the fold changes for the molecular signature in the original dataset.

Hierarchical clustering analysis and data transformation for microarray analysis were performed using Cluster version 2.11 (235). Microarray data visualization using
heatmaps was prepared using Java Treeview (236), where red depicts an increased expression and green depicts a decreased expression in a given gene.

**Preparation of samples for Quantitative Real-Time PCR analysis**

For *src* analysis SaOS-2, U2 OS and SK-LMS-1 cells were treated with 30, 100, 300 and 1000 nM of dasatinib or DMSO for six hours. Cells were washed with ice cold 1x PBS, RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The aqueous phase containing the RNA separated from the TRIzol reagent was further purified using the RNeasy cleanup procedure (Qiagen Inc., Valencia, CA). The quantitative Real-Time PCR analysis for *histone H3*, *FAF1*, *α-catenin*, *δ-catenin*, *ephrin-A1* and *dapper* was completed using the same RNA samples used for the microarray analysis.

**cDNA reactions**

Reverse Transcriptase reactions were random hexamer-primed using Applied Biosystems’ (Foster City, CA) High Capacity cDNA Archive Kit. (All Reverse Transcriptase reactions were done at the same time so that the same reactions could be used for all gene studies.) For the construction of standard curves, serial dilutions of pooled sample RNA were used (50, 10, 2, 0.4, 0.08, and 0.016 ng) per reverse transcriptase reaction. One “no RNA” control was included. Additionally, one ‘no reverse transcriptase’ control was included for the standard curve and for each sample.
**Real-Time PCR reactions**

TaqMan® Gene Expression Assays (Applied Biosystems) were used. The assay primer & probe sequences are proprietary. The probe is labeled with 6-carboxy-fluorescein as the reporter on the 5’ end, and a non-fluorescent quencher plus a minor-groove binder on the 3’-end. Each assay is supplied as a 20X mix of primers and probe. Real-time quantitative PCR analyses were performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). All standards, the no template control (H2O), and the no amplification control (Bluescript plasmid) were tested in quadruplicate wells (2 wells/plate x 2 plates). All samples were tested in triplicate wells. The no RT controls were tested in duplicate wells. PCR was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) using 2 µl of cDNA and 1X primers & probe in a 20-µl final reaction mixture. After a 2-min incubation at 50°C, AmpliTaq Gold was activated by a 10-min incubation at 95°C, followed by 40 PCR cycles consisting of 15 s of denaturation at 95°C and hybridization of probe and primers for 1 min at 60°C. Data were analyzed using SDS software version 2.2.2 and exported into an Excel spreadsheet. The 18s data were used for normalizing the gene values - ng gene/ng 18s per well.

**Personal health identifier**

Tissues from patients treated at the Sarcoma clinic and collected by the tissue procurement lab at the H. Lee Moffitt Cancer Center were used for this study. The specimens were handled and stored by the tissue procurement lab under the supervision of Dr. T. Hoover. Specimens were released for RNA extraction and microarray analysis.
after the removal of all PHIs. The specimens were coded and could not be linked to the donor any individual involved in the analyses of the said samples.

**Involvement of human subjects**

No procedures were performed on patients. Only pathologic material that was previously collected for research purposes and that was not necessary for a complete pathologic diagnosis was utilized in this study. A separate informed consent beyond what is routine for surgery at H. Lee Moffitt Cancer Center was not necessary.
Introductory Preclinical Data

Studies have demonstrated that STAT proteins participate in essential cellular functions including cellular immune function, development, differentiation and proliferation (237-242). STATs are a family of latent cytoplasmic transcription factors that associated with RTKs and NRTKs. Upon association with activated tyrosine kinases and activation by tyrosine phosphorylation they can either homodimerize or heterodimerize with other activated STAT proteins via SH2 domain-phosphotyrosine interacting with other STAT family members. Activated STAT dimers translocate to the nucleus and regulate gene transcription by binding to elements within gene promoters (243, 244).

An accumulation of evidence has demonstrated a critical role of STAT3 in the molecular pathology of cancer, including tumor formation (245, 246). Constitutive activation of STATs has been shown to play a significant role in the malignant transformation of cells (245, 247, 248). Specifically, constitutive activation of STAT3 is associated with transformation by v-Src and other viral oncoproteins that activate tyrosine kinase signaling pathways (187, 217, 249, 250) and is required for the transcription of genes involved in v-Src-induced cellular transformation (186, 251).

STATs have been demonstrated to be constitutively activated in epithelial and hematopoietic human cancers. However, despite overwhelming evidence to suggest a potential role of STATs in sarcomagenesis, few direct studies have been completed to
evaluate the role of STAT3 activation in human sarcomas. The most significant evidence to imply a role for STATs in sarcomagenesis is the overexpression and/or mutation of several TKs in sarcomas. Recall that PDGFR, c-KIT, IGFR-1 and VEGFR have been shown to be overexpressed in many sarcomas of various histologies. STATs are involved in transducing TK signaling for all of these pathways under normal and malignant conditions. Furthermore, Src, another essential protein involved in activating STATs and eliciting the activation of the aberrantly activated TKs in sarcomas also activates STAT3. Src was originally identified as the viral oncoprotein responsible for RSV, however the role of Src has yet to be established in human sarcomas. Therefore, the evaluation of the Src-STAT3 signaling pathway in human sarcoma cell lines was initiated.

Signaling by STAT proteins is associated with activation of Src tyrosine kinase (187, 249), thus Immunohistochemistry (IHC) was utilized to determine activation status of STAT3 in human sarcoma specimens and STAT3 DNA-binding activity in nuclear extracts from human sarcoma cell lines. Pharmacological inhibitors of Src were used to evaluate its role in STAT3 activation in human sarcoma cell lines.
Results

*Expression of SFKs in sarcoma cell lines*

RT-PCR was performed to determine which SFKs were expressed in eight sarcoma cell lines. Of the 11 recognized SFK members, three including Src, Fyn and PTK2 were expressed in all eight cell lines examined (Fig. 5A-H; Table 5). Yes1 was expressed in all but the HT-1080 cell line (Fig. 5G). Hck was not expressed in any of the cells lines, while only barely detectable levels of Lck were expressed in U2 OS cells (Fig. 5D). The remaining SFK members were expressed in some cell lines, but not others without an apparent preference for tumor type, i.e. STS or OSA.

*STAT3 is activated in human sarcomas and sarcoma cell lines*

Immunohistochemistry for the activated form of STAT3 protein was performed on human sarcoma specimens using antibodies specific for phosphorylated STAT3 Tyr705 (pSTAT3). STAT3 is activated upon phosphorylation of Tyr705, which induces dimerization, translocation to the nucleus and DNA binding (248). Results show activated STAT3 in sarcomas of diverse subtypes (Fig. 6), including high grade OSA (A), pleomorphic LPS (B) and pleomorphic, undifferentiated high grade sarcoma (C). Since activation of STAT3 was found in the majority of sarcoma tissues examined (Fig. 6D), we determined the status of STAT3 activation in seven sarcoma cell lines using EMSA. Results show STAT3 was activated in all seven cell lines to varying degrees (Fig. 6E); MG-63, an OSA cell line, had the greatest and HT1080, a FBS cell line, had the least amount of STAT3 activation.
Figure 5. Evaluation of SFK expression in human sarcoma cell lines. The expression of SFKs were determined in eight sarcoma cell lines using the SFK Superarray multiGene-12 RT-PCR Profiling Kit. The kit was performed on eight cell lines and the PCR products were resolved on 1% agarose gels for SaOS-2 (A), LM-2 (B), LM-7 (C), U2 OS (D), MG-63 (E), SK-LMS-1 (F), HT1080 (G) and SW-872 (H). The lane numbers correspond to the specific SFK for that well and the numbering system for can be found on Table 5.
Table 5. SFK gene expression status in human sarcoma cell lines

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| SaOS-2    | x x     | x x     | x x     | x x     | x x     | x x     | x x     
| LM-2      | x x     | x x     | x x     | x x     | x x     | x x     | x x     
| LM-7      | x x     | x x     | x x     | x x     | x x     | x x     | x x     
| U 2 OS    | x x     | x x     | x x     | x x     | x x     | x x     | x x     
| MG-63     | x x     | x x     | x x     | x x     | x x     | x x     | x x     
| SK-LMS    | x x     | x x     | x x     | x x     | x x     | x x     | x x     
| HT1080    | x x     | x x     | x x     | x x     | x x     | x x     | x x     
| SW872     | x x x   | x x x   | x x x   | x x x   | x x x   | x x x   | x x x   

Table 5. PCR was performed for 11 SFK family members in eight sarcoma cell lines. The presence of an ‘x’ on the table denotes expression of the corresponding SFK. The SFK genes are listed at the top of the table, the number in parentheses corresponds to the lane number in Figure 6; panels A-H.
D

[Image showing the pStat3 Expression Index graph for different types of sarcoma: Osteosarcoma, Chondrosarcoma, EWS, MPNST, Angiosarcoma, Undifferentiated High Grade Sarcoma, Liposarcoma, and Leiomyosarcoma. Each type of sarcoma is represented by a horizontal bar corresponding to different expression indices.]
Figure 6. STAT3 activation status in human sarcoma tissues and cell lines. A-C, immunohistochemistry for p-STAT3 (Y705) reveals that Src is activated in human sarcoma tissues; high grade OSA (A), pleomorphic LPS (B) and pleomorphic, undifferentiated high grade sarcoma (C). D, STAT3 is activated in several diverse sarcomas, this graph depicts the average intensity of STAT3 phosphorylation as measured by IHC. E, Cell-free extracts were prepared from untreated cells grown in 10% FBS and EMSA was performed to determine the activation status of STAT3 in several sarcoma cell lines. Supershift was completed on all cell lines in previous experiments to prove that STAT3, specifically, is activated in the cell lines. Supershift was only completed on SaOS-2 in lane one of this experiment and is denoted by the *.
**PD180970 inhibits Src and STAT3 signaling in human sarcoma cell lines**

PD180970 has previously been shown to directly inhibit the kinase activity of purified Src protein *in vitro* with an IC$_{50}$ of 16.8 nM (252). To evaluate the effect of PD180970 on Src kinase activity in intact sarcoma cells, we treated the SK-LMS-1, SaOS-2, LM2 and U2 OS cell lines with escalating doses of PD180970 (125, 250 and 500 nM) for 24 h. Western blot analysis was performed to evaluate p-Src (Tyr419) levels and EMSA was performed to determine the effect on STAT3 activation. Dose-response results for p-Src are shown in Figure 7A for a representative cell line (SK-LMS-1) and STAT3 activation for SK-LMS-1, SaOS-2 and LM2 are shown in Figure 7B. Src phosphorylation is diminished with 125 nM and completely inhibited with 250 nM PD180970. The kinetics of STAT3 inhibition is not as straightforward as the Src response to PD180970. EMSA shows that STAT3 activation is partially inhibited with all doses of PD180970 in three of the four cell lines displayed (Fig. 7B). SaOS-2 is the most sensitive with STAT3 inhibition markedly decreased with 125 nM PD180970. SK-LMS-1 and LM2 demonstrate maximum inhibition of STAT3 activation with 500 nM PD180970. U2 OS cells had no response to PD180970 as measured by Src phosphorylation and STAT3 activation. The activity of Src (data not shown) and STAT3 remained constitutively activated at all doses of PD180970 (Fig. 7B). Time course experiments were performed to determine the inhibitory kinetics of PD180970 on STAT3 activation. As shown in representative results with SK-LMS-1 cells, inhibition of STAT3 activation is reduced at 3 h and is completely inhibited at 24 h with 500 nM treatment of PD180970 (Fig. 8A). However, STAT3 activation increases at 36 h and later time points, although activation does not return to untreated levels.
Figure 7. PD180970 inhibits Src and STAT3 signaling in sarcoma cell lines. 

**A**, SK-LMS-1 cells were treated with PD180970 in a dose-dependent manner for 6 h. Cell-free extracts were immunoblotted with antibodies specific to p-Src (Y419) and β-actin. 

**B**, SK-LMS-1, SaOS-2, LM-2 and U2 OS cells were treated with PD180970 in a dose response for 24 h. EMSA was performed to evaluate STAT3 activation. Western blot analysis and EMSA were performed as described. DMSO was used as a vehicle control in all experiments.
PD180970 inhibits cell viability and induces apoptosis in sarcoma cell lines

To determine the effect of PD180970 on sarcoma cell survival over time, we performed growth curve analyses in cell lines treated with 500 nM PD180970 in a time course. These assays suggested that the sarcoma cell lines responded to PD180970 by induction of apoptosis in a time-dependent manner (Fig. 8B). SaOS-2 cells experienced reduced viability with increased exposure to 500 nM PD180970. By 36 h, less than 55% of the cells were viable (p< 0.05) and by 72 h less than 10% of the cells were viable (p<0.001). To further validate the induction of apoptosis, Western blot analysis was performed for PARP cleavage. SK-LMS-1 and SaOS-2 cells were treated with 500 nM of PD180970 increasing periods of time and PARP cleavage was evaluated in both cell lines (Fig. 8C). PARP cleavage, an indicator of apoptosis, was evident in both cell lines after 8 h of treatment and increased with time. Moreover, TUNEL assays performed on SK-LMS-1, SaOS-2 and LM2 cells confirmed that increasing numbers of the cells underwent apoptosis 48 h after treatment with 500 nM of PD180970 (Fig. 9A-C respectively). Therefore, PD180970 induces apoptosis in sarcoma cell lines with doses that correspond to the inhibition of Src and STAT3 activation by PD180970.
Figure 8. PD180970 inhibits viability and induces apoptosis in sarcoma cell lines.  

A, PD180970 inhibits STAT3 activation in a time-dependent manner. SK-LMS-1 cells were treated with 500 nM PD18097 for increasing periods of time. Cell-free extracts were utilized for EMSA to determine STAT3 activation status. B, SaOS-2 were treated with 500 nM PD18097 in a time course and cell viability was determined by trypan blue exclusion assays in triplicate. C, SK-LMS-1 and SaOS-2 cells were treated with 500 nM PD18097 in a time course. Cell-free extracts were immunoblotted with antibodies specific to PARP to measure induction of apoptosis. DMSO was used as vehicle control for all experiments.
Figure 9. PD180970 induces apoptosis in sarcoma cell lines as measured by TUNEL. A-C, apoptosis was further verified by TUNEL assay as described. SK-LMS-1, SaOS-2 and LM-2 cells were plated in 12-well tissue culture plates and treated with DMSO or 500 nM PD180970 for 48 h. After completing the TUNEL assay, cells were visualized using light microscopy and photographed at 20x magnification.
SKI-606 does not inhibit Src-STAT3 signaling in human sarcoma cell lines

SKI-606 has previously been shown to directly inhibit the kinase activity of purified Src protein in vitro with an IC50 of 3.8 nM (253) and 100 nM for intact cells (254). To evaluate the effect of SKI-606 on Src kinase activity and STAT3 activation in intact sarcoma cells, we treated several sarcoma cell lines with escalating doses of SKI-606 (300, 1000, 3000 and 10,000 nM) for 24 h. Western blot analysis was performed to evaluate p-Src (Y419) levels and EMSA was performed to determine the effects on STAT3 activation. There were no apparent effects on pSrc (Y419) in any of the cell lines evaluated (data not shown). Furthermore, dose-response results for STAT3 activation for SK-LMS-1 and U2 OS are shown in Figure 10. There was no inhibition of STAT3 activation by SKI-606 in any of the cell lines examined. Furthermore, there were no observed anti-proliferative or pro-apoptotic effects from SKI-606 at any of the doses tested.

Figure 10. SKI-606 does not inhibit Src-STAT3 signaling in sarcoma cell lines. SK-LMS-1 and U2 OS cell were treated with SKI-606 in a dose-response for 24 h. EMSA was performed to measure STAT3 activation in response to SKI-606 treatment. DMSO was used as vehicle control.
Dasatinib does not inhibit Src-STAT3 signaling in human sarcoma cell lines

Dasatinib has previously been shown to directly inhibit the enzymatic activity of Src with an IC$_{50}$ of 0.5 nM (231). The effects of Src activity are described in the next chapter of this dissertation. However, to evaluate the effect of dasatinib on STAT3 activation in sarcoma cells, several sarcoma cell lines were treated with escalating doses of dasatinib (30, 100, 300 and 1000 nM) for 6 h. EMSA was performed to determine the effects on STAT3 activation six cell lines. There was no inhibition of STAT3 activation by dasatinib in any of the cell lines examined Fig. 11A. Western blot analysis was performed to confirm these observations, Figure 11B and C show that pSTAT3 expression does not decrease with increased dasatinib concentration in SaOS-2 and U 2 OS cell lines, respectively. Furthermore, there were no observed effects of STAT3 activation by dasatinib in SK-LMS-1 cell lines when the doses escalated to 3 and 10 µM for 24 h (Figure 11D).
Figure 11. STAT3 signaling is independent of Src kinase activity in human sarcoma cell lines. 

A, SaOS-2, LM-2, U 2 OS, MG-63, SK-LMS-1 and HT1080 were treated with dasatinib in a dose response for 6 h. EMSA was performed to evaluate STAT3 activation status in response to treatment with dasatinib. 

B and C, SaOS-2 and U 2 OS cells were treated with escalating doses of dasatinib for 6 h and cell free extracts were immunoblotted for pSTAT3 (Y705) and β-actin as loading control. 

D, SK-LMS-1 cells were treated with dasatinib in a dose response that achieved higher doses of dasatinib for 24 h. EMSA analysis was performed to evaluate STAT3 activation status in response to treatment with dasatinib.
Discussion

Constitutive activation of STAT3 has been observed and demonstrated to play an essential role for the tumorigenesis in many solid tumor and hematopoietic malignancies (195, 196, 245, 246). The status of STAT3 activation in sarcomas has not been explored previously. Our results show that STAT3 is constitutively activated in many of the human sarcoma specimens analyzed and most of the sarcoma cell lines examined. To elucidate the role of STAT3 activation in human sarcoma cell lines, we investigated the activity of three different Src kinase inhibitors. We have shown the PD180970 inhibits Src and STAT3 signaling in several of the cell lines utilized. Although the IC₅₀ for STAT3 inhibition is markedly higher than the IC₅₀ required to inhibit Src activity for most of the responsive cell lines (Fig. 7). However, cell viability was decreased and apoptosis was induced by 500 nM PD108970 treatment in a time-dependent fashion. Furthermore, the dose of PD180970 corresponded to complete inactivation of Src and STAT3 kinases in all responsive cell lines.

These data highlight the potential promise of employing a Src or STAT3 inhibitors for the treatment of sarcomas. There is little incentive to perform additional experiments with PD180970 because the stability of this compound is limited and obtaining reproducible data is difficult. This compound also has little to no future in the clinic as it is only soluble in DMSO and is highly nonspecific. In addition, several more stable, more specific, bioavailable Src kinase inhibitors with more promising clinical futures have been made available. Two such Src kinase inhibitors are SKI-606 and
dasatinib were further investigated to determine a more precise role for Src and STAT3 activity in sarcomas.

The Src kinase inhibitor, SKI-606, is currently undergoing several phase 2 clinical trials for malignancies including breast, pancreatic, colon and non-small cell lung cancers and chronic myelogenous leukemia (255). We evaluated the activity of this compound in sarcoma cell lines to investigate the effects of Src and STAT3 signaling. To our disappointment, SKI-606 had no inhibitory activity on either of these proteins in sarcomas. There were no apparent proliferation or survival effects generated by exposure to this compound either. One possible explanation for these results may be that the compound is not crossing the cell membrane and gaining access to the cell. Src kinase may also have a greater affinity for PD180970 or dasatinib, which may further explain the lack of response to SKI-606.

Dasatinib was the last Src inhibitor to be obtained and evaluated, interestingly enough it was also the compound that exhibited the most potential as a possible therapeutic option for sarcomas. However, the promising anti-tumor effects of dasatinib do not appear to be mediated via a Src-STAT3 pathway. Dasatinib was shown to potentially inhibit Src activity in sarcoma cell lines, as will be more thoroughly demonstrated in the following chapter. Surprisingly, inhibition of Src activity by dasatinib had no effect on STAT3 activation in sarcoma cell lines (Fig. 11). These results do not rule out a possible role for constitutive STAT3 activation in sarcomas, but suggests that the Src and STAT3 signaling pathways may be uncoupled in sarcomas. The data generated using PD180970 suggested a higher IC50 was required to inhibit STAT3 as
compared to Src activity, suggesting that Src and STAT3 may be cross-talking with other pathways in sarcoma cell lines. In spite of this, these results support the possibility of a dependence of Src activity for survival in sarcomas. Therefore, we next set out to determine the role of Src activity in sarcomas by investigating other Src dependent pathways.
Dasatinib Inhibits Migration and Invasion in Diverse Human Sarcoma Cell Lines and Induces Apoptosis in Bone Sarcoma Cells Dependent on Src Kinase for Survival

One potential molecular target for sarcoma treatment is the Src tyrosine kinase. Dasatinib, a small-molecule inhibitor of Src kinase activity, is a promising cancer therapeutic agent with oral bioavailability. Dasatinib exhibits anti-tumor effects in cultured human cell lines derived from epithelial tumors including prostate and lung carcinomas. However, the action of dasatinib in mesenchymally-derived tumors has yet to be demonstrated. Dasatinib was originally selected as a Src kinase inhibitor and then shown to inhibit Bcr-Abl as well as other tyrosine kinases. There have been several studies demonstrating the activity of dasatinib against Bcr-Abl-positive leukemic cell lines as well as epithelial tumor cell lines (43-48). In addition, early phase clinical trials have established the safety and efficacy of dasatinib for treatment of imatinib-resistant chronic myelogenous leukemia patients. However, the responses and mechanisms of action of dasatinib in mesenchymally-derived tumor cell lines have not been described previously. We report that dasatinib inhibits Src and downstream FAK signaling at nanomolar concentrations, blocks cell migration and invasion in many diverse human sarcoma cell lines and induces apoptosis in bone sarcomas. Furthermore, knockdown of Src expression by siRNA in bone sarcoma cells also induces apoptosis, suggesting that the observed response to dasatinib in these cells is conveyed through inhibition of Src-mediated signaling. Together, these findings indicate that dasatinib is a promising
therapeutic agent for preventing growth and metastasis of a wide diversity of soft-tissue and bone sarcomas. Based on our previous findings of Src activation in human sarcomas, we evaluated the effects of dasatinib in twelve cultured human sarcoma cell lines derived from bone and soft-tissue sarcomas.
Results

Src kinase is activated in human sarcomas and sarcoma cell lines

Immunohistochemistry for activated Src protein was performed on human sarcoma specimens for activated Src protein using antibodies to phospho-Src (p-Src). Levels of p-Src on tyrosine residue 419 (Y419) due to autophosphorylation reflect Src kinase activities in intact cells and tissues. Results show activated Src in sarcomas of diverse subtypes (Fig. 12), including leiomyosarcoma (A), high-grade OSA (B), and LPS (C). Since autophosphorylated Src was found in a majority of the human sarcomas examined, including diverse soft-tissue and bone sarcomas (data not shown), we determined the level of Src activation in a panel of human sarcoma cell lines by Western blot analysis for p-Src (Y419) and total Src protein levels. Src was detectably activated in all but one (HT-1080) of the cell lines examined, albeit to different extents (Fig. 12D). Total Src protein expression does not correlate with levels of phosphorylated Src in every case, indicative of different levels of Src kinase activation among the sarcoma cell lines. In addition, the level to which Src kinase is activated (p-Src levels) does not correlate with specific sarcoma histological sub-types (Fig. 12 and Table 6).
Figure 12. Src kinase is activated in human sarcoma tissues and cell lines. A–C, immunohistochemistry for p-Src (Y419) reveals that Src is activated in human sarcoma tissues (leiomyosarcoma A, high grade osteosarcoma B and liposarcoma C). D, Src is activated in all but one (HT-1080) of the cell lines utilized for these experiments. Cell-free extracts were prepared from untreated cells grown in 10% FBS and immunoblotted with antibodies specific for p-Src (Y419), total Src or β-actin.
Dasatinib inhibits Src kinase activity in human sarcoma cell lines

Dasatinib has previously been shown to directly inhibit the kinase activity of purified Src protein in vitro with an IC₅₀ of 3 nM (223). To evaluate the effect of dasatinib on Src kinase activity in intact sarcoma cells, we treated the above cell lines with escalating doses of dasatinib (30, 100, 300 and 1000 nM) for 6 h and Western blot analysis was performed to evaluate p-Src levels. Dose-response results for the two representative cell lines (SaOS-2 and U 2 OS) are shown in Figure 13 (A and B), the IC₅₀ values for inhibition of p-Src by dasatinib range from 3 to 68 nM for all cell lines analyzed (summarized in Table 6). Time course experiments were performed to determine the kinetics at which Src phosphorylation is inhibited by dasatinib. As shown in representative results with U 2 OS cells, inhibition of Src phosphorylation is complete by 15 min following treatment with 100 nM of dasatinib and persists for at least 24 h (Fig. 13C). Interestingly, total Src protein expression was increased in a dose- and time-dependent manner in a subset of the cell lines treated with dasatinib. In particular, all but one bone sarcoma cell line (MG-63) produced increases in total Src protein expression, yet this effect was not observed in the soft-tissue sarcoma cell lines (Fig. 13A-C, and data not shown). However, this increased protein expression of Src was not accompanied by an increase of c-Src mRNA, as shown in both cell lines which an increased expression of total Src was and was not observed (Fig. 14). These data suggest a positive feedback mechanism for compensation of Src kinase inhibition with increased levels of Src protein expression in the bone sarcoma cells.
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Figure 13. Dasatinib inhibits Src activation and downstream signaling in sarcoma cell lines. A and B, SaOS-2 and U 2 OS cells were treated with dasatinib in a dose-dependent manner for 6 h. Cell-free extracts were immunoblotted with antibodies specific to p-Src (Y419) and total Src. C, U 2 OS cells were treated with 100 nM of dasatinib in a time-dependent manner. Western blot analysis was performed as described. DMSO was used as a vehicle control and β-actin was immunoblotted for as a loading control in all experiments. D, dasatinib specifically blocks tyrosyl phosphorylation of FAK (Y576/577, Y925) and 130CAS (Y410), but not FAK Y397. SaOS-2 cells were treated with dasatinib for 6 h in a dose-dependent manner. Cell-free extracts were immunoblotted with antibodies specific to p-FAK (Y397, Y576/577, Y925), total FAK, p130CAS and p-p130CAS (Y410).
Figure 14. Dasatinib does not induce c-Src mRNA expression. Quantitative RT-PCR was performed on SaOS-2, U 2 OS and SK-LMS-1 cells to determine the effect of dasatinib on c-Src mRNA expression. Cells were treated with dasatinib in a dose-response and RNA was isolated, purified and qRT-PCR for c-Src was performed as described.
Dasatinib selectively blocks Src downstream signaling

Src kinase has been shown to regulate cellular activities through a number of downstream signaling pathways. One such pathway is FAK, a non-receptor tyrosine kinase found to be increased in a variety of epithelial cancers including those arising from prostate, cervical and colon (256-259). Furthermore, increased FAK expression is associated with tumor progression in a mouse model of skin carcinogenesis (200). FAK, in turn, has been implicated in the activation of CRK-associated substrate, p130\textsuperscript{CAS}, which together with Src and FAK plays a vital role in cell adhesion, migration, proliferation and survival (209). To investigate the effect of dasatinib on these Src downstream signaling pathways, sarcoma cell lines were treated in culture with escalating doses of dasatinib for 6 h. As representative results using SaOS-2 cells, Figure 13D shows Western blot analysis performed using antibodies to total FAK protein, phosphorylated FAK (Y397, Y576/Y577, and Y925), p130\textsuperscript{CAS} and phosphorylated p130\textsuperscript{CAS} (Y410). The IC\textsubscript{50} values for inhibition of phosphorylated FAK (Y576/Y577 and Y925) and p130\textsuperscript{CAS} were between 30 to 100 nM, consistent with the IC\textsubscript{50} values for inhibition of Src kinase activity in these cells (Fig. 13 and Table 6). FAK autophosphorylation (Y397) was not substantially inhibited until higher doses of dasatinib (1000 nM), indicating that dasatinib does not directly inhibit FAK kinase activity. Surprisingly, total p130\textsuperscript{CAS} protein was diminished with dasatinib treatment while total FAK protein was not affected (Fig. 13D), suggesting that p130\textsuperscript{CAS} protein is subject to negative feedback regulation in these cells. By contrast, dasatinib did not inhibit STAT3 signaling in sarcoma cell lines (data not shown), another signaling pathway that has been shown to act downstream of Src in cells of other tumor types.
Thus, dasatinib selectively blocks FAK and p130^{CAS} signaling downstream of Src in sarcoma cell lines.

**Dasatinib blocks cell motility and invasion by sarcoma cells**

Both FAK and p130^{CAS} activity are involved in regulating cell migration and invasion downstream of Src kinase. The effect of dasatinib on cell migration was evaluated using “wound healing” assays (by scratching cell monolayers with a pipette tip) and treating with drug. Cells were plated in 0.1% serum medium prior to inducing the wound to ensure that migration rather than cell growth was measured. The width of the wound was determined at T0 and then cells were treated with escalating doses of dasatinib in 0.1% serum medium for 24 h. Representative results are shown with the SaOS-2 cells, which were digitally photographed and the width of denuded area in the wound was measured in pixels (Fig. 15A and B). Wound healing was dramatically inhibited by dasatinib in a dose-dependent manner, with detectable inhibition at 30 nM and substantial inhibition at 100 nM dasatinib. To evaluate the effect of dasatinib on cell invasion, SaOS-2 and U 2 OS cells were treated with dasatinib in a dose-response manner for 22 h in Matrigel Invasion Chambers. Dasatinib significantly inhibited cellular invasion in both cell lines (Fig. 15C). The SaOS-2 cell line was more sensitive to inhibition of invasion by dasatinib compared to the U 2 OS cell line. The IC_{50} values for inhibition of tumor cell invasion in this assay ranged from 30 to 100 nM. These IC_{50} values for blockade of cell migration and invasion are consistent with the IC_{50} values for inhibition of Src kinase as well as downstream FAK and p130^{CAS} signaling (compare with Table 6 and Fig. 13D).
A

T₀

DMSO

30 nM

100 nM

300 nM

1 µM

B

Dasatinib, nM

Place

T₀ 0 30 100 300 1000

dasatinib, nM
Figure 15. Dasatinib inhibits cell motility and invasion. A, wound healing assays were performed to determine the effects of dasatinib on inhibiting cell migration. SaOS-2 cells were plated in 12-well tissue culture plates, grown to confluency and serum-starved overnight in medium containing 0.1% FBS. Wounds were introduced on cell monolayers using a pipette tip. Cells were washed with 1x PBS to remove non-adherent cells and treated with DMSO or dasatinib in a dose-dependent manner for 24 h. Cell migration was visualized at 10x magnification by light microscopy and photographed with a digital camera. B, width of voided area versus dasatinib dose concentration was graphed to express the degree of inhibition of cell migration. The number of pixels within the denuded area were the units used to demonstrate inhibition of cell migration induced by dasatinib (*p<0.001, n=3). C, matrigel Invasion Chambers were used to measure dasatinib inhibition of cellular invasion. SaOS-2 (solid bars) and U 2 OS (shaded bars) were treated with dasatinib in a dose-dependent manner. Cells were diluted in 500 μL of serum-free medium and placed over the inner chamber of the insert in a 24-well tissue culture plate and 500 μL of complete medium was placed in the lower chamber of the insert. After incubating for 22 h, cells that invaded through the Matrigel were stained, visualized using light microscopy, photographed and counted. Each experiment was performed in triplicate. The mean values of invasive cells were graphed versus dasatinib concentration (*p<0.001, **p<0.01, n=3). To confirm that invasion was measured rather than induction of apoptosis, trypan blue exclusion assays were performed with SaOS-2 and U 2 OS cells treated in a dose-dependent manner with dasatinib for 24 h. Greater than 90% of the cells were viable after dasatinib treatment (data not shown).
Dasatinib induces apoptosis of bone sarcoma cell lines

To determine the effect of dasatinib on sarcoma cell survival, we performed growth curve analyses in cell lines treated with increasing concentrations of dasatinib. These assays suggested that the subset of cell lines derived from bone sarcomas responded to dasatinib by induction of apoptosis in a dose-dependent manner (data not shown). To further validate the induction of apoptosis in this subset of cell lines, Western blot analysis was performed for apoptotic markers. SaOS-2 and U 2 OS cells were treated with escalating doses of dasatinib for 72 h, and PARP cleavage and XIAP expression were evaluated in both cell lines (Fig. 16A and C). PARP cleavage, an indicator of apoptosis, is evident at 30 nM dasatinib and increased with escalating doses of dasatinib. Furthermore, expression of XIAP, an inhibitor of apoptosis, was diminished by dasatinib treatment with IC50 values ranging from 30 nM to 100 nM (Fig. 16A and C). A time-course analysis with 100 nM dasatinib was performed in the SaOS-2 cell line to determine when apoptosis was induced as measured by PARP cleavage. PARP cleavage is evident as early as 6 h following treatment with dasatinib and increased with time (Fig. 16B). Moreover, TUNEL assays performed on SaOS-2 cells confirmed that increasing numbers of the cells were undergoing apoptosis by 48 h after treatment with escalating doses of dasatinib (Fig. 16D). Therefore, dasatinib induces apoptosis in the bone sarcoma subset with IC50 values corresponding to those of inhibition of Src kinase and downstream signaling by dasatinib (Table 6).
A

<table>
<thead>
<tr>
<th>DMSO 30 100 300 1000 nM</th>
<th>XIAP</th>
<th>PARP</th>
<th>PARP cleavage</th>
<th>β-Actin</th>
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B

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<th>PARP cleavage</th>
<th>β-Actin</th>
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C

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<th>DMSO 30 100 300 1000 nM</th>
<th>XIAP</th>
<th>PARP</th>
<th>PARP Cleavage</th>
<th>β-Actin</th>
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</table>
Figure 16. Dasatinib induces apoptosis in bone sarcoma cell lines. A and C, dasatinib induces apoptosis in a dose-dependent manner. SaOS-2 and U-2 OS cells were treated with dasatinib for 72 h with escalating doses. Cell-free extracts were immunoblotted with antibodies specific to XIAP and PARP. B, SaOS-2 cells were treated with 100 nM of dasatinib in a time-dependent manner and immunoblotted with an antibody specific for PARP. D, apoptosis was further verified by TUNEL analysis as described. SaOS-2 cells were plated in 12-well tissue culture plates and treated with dasatinib for 48 h in a dose-dependent manner. After completing the TUNEL assay, cells were visualized using light microscopy and photographed at 20x magnification.
To determine if inhibition of Src kinase by dasatinib is sufficient to induce apoptosis in the bone sarcoma cell lines, we transfected these cell lines with siRNA to c-Src. Two representative bone sarcoma cell lines, SaOS-2 and U2 OS, underwent induction of apoptosis in a dose-dependent manner as measured by PARP cleavage in response to siRNA against c-Src but not to control siRNA. Src protein expression was inhibited by transfection with 50 nM and 100 nM siRNA against c-Src, corresponding to induction of PARP cleavage in both cell lines (Fig. 17A and B). MG-63, an OSA cell line that does not undergo apoptosis when treated with dasatinib, also does not undergo apoptosis when transfected with siRNA to Src (data not shown). These data demonstrate that a subset of the bone sarcoma cell lines rely on Src kinase for survival, indicating that inhibition of Src kinase activity by dasatinib is sufficient to induce apoptosis in these cells.
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<tr>
<td>PARP Cleavage</td>
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<tr>
<td>β-actin</td>
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<tr>
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<tr>
<td>β-actin</td>
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</tbody>
</table>
Figure 17.  Src is required for bone sarcoma cell line survival.  A and B, siRNA to c-Src induces apoptosis in bone sarcoma cell lines. SaOS-2, A, and U 2 OS, B, cells were plated in 6 cm tissue culture plates, transfected with 50 and 100 nM of siRNA to c-Src (si Src) and harvested after 72 h. Cell-free extracts were immunoblotted with antibodies specific for Src and PARP to measure the efficiency of knockdown by siRNA and to determine if apoptosis was induced upon depletion of Src protein expression. C, Src activation and signaling are inhibited by dasatinib. Inhibition of Src signaling by dasatinib prevents cellular migration and invasion in sarcoma cell lines. Upon inhibition of Src phosphorylation or c-Src expression, a sub-set of bone sarcoma cell lines undergo an induction of apoptosis.
Table 6. Summary of cell line IC\textsubscript{50} values and responses to dasatinib

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Tumor Type</th>
<th>pSrc (Y419) Expression</th>
<th>IC\textsubscript{50}, nM</th>
<th>Induction of Parp Cleavage, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaOS-2</td>
<td>Osteosarcoma</td>
<td>++</td>
<td>46</td>
<td>65</td>
</tr>
<tr>
<td>LM2</td>
<td>Osteosarcoma</td>
<td>++</td>
<td>26</td>
<td>35</td>
</tr>
<tr>
<td>LM7</td>
<td>Osteosarcoma</td>
<td>++</td>
<td>68</td>
<td>24</td>
</tr>
<tr>
<td>U 2 OS</td>
<td>Osteosarcoma</td>
<td>+++</td>
<td>57</td>
<td>4</td>
</tr>
<tr>
<td>MG-63</td>
<td>Osteosarcoma</td>
<td>+++</td>
<td>28</td>
<td>58</td>
</tr>
<tr>
<td>SK-ES-1</td>
<td>Ewing’s Sarcoma</td>
<td>+++</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>TC-71</td>
<td>Ewing’s Sarcoma</td>
<td>+</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>SK-LMS-1</td>
<td>Leiomyosarcoma</td>
<td>+++</td>
<td>46</td>
<td>44</td>
</tr>
<tr>
<td>HT-1080</td>
<td>Fibrosarcoma</td>
<td>-</td>
<td>N/R</td>
<td>N/R</td>
</tr>
<tr>
<td>A673</td>
<td>Rhabdomyosarcoma</td>
<td>++++</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td>RD</td>
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<tr>
<td>RD18</td>
<td>Rhabdomyosarcoma</td>
<td>+</td>
<td>50</td>
<td>277</td>
</tr>
</tbody>
</table>

\(+/−\) depicts relative p-Src (Y419) expression

N/R No Response

Table 6. Summary of responses to dasatinib in human sarcoma cell lines. With the exception of one soft-tissue sarcoma cell line (HT1080) all of the cell lines examined respond to dasatinib by inhibition of Src phosphorylation on Y419 and migration at IC\textsubscript{50} values consistent with Src kinase inhibition. A subset of bone sarcoma cell lines respond to dasatinib treatment by induction of apoptosis. Induction of apoptosis was not observed in the soft-tissue sarcomas cell lines and one osteosarcoma (MG-63) cell line.
Discussion

After nearly a century since the discovery of the Rous sarcoma virus, which subsequently was shown to induce sarcomas by capturing and mutationally activating the cellular gene encoding the Src tyrosine kinase, targeted Src kinase inhibitors are now entering clinical trials for solid tumors. Sarcomas comprise a highly diverse set of human tumors that frequently occur among pediatric cancer patients and for which there are limited treatment options. Based on our observation of Src kinase activation in sarcoma clinical specimens, we sought to determine the action of dasatinib, a potent and orally bioavailable inhibitor of Src kinase, on human sarcoma cell lines.

Our findings demonstrate that dasatinib inhibits Src kinase activity, as measured by autophosphorylation at Tyr419, in a dose-dependent manner in sarcoma cells. Furthermore, in 11 out of 12 sarcoma cell lines examined dasatinib inhibits cell migration and invasion. The single cell line that did not respond to dasatinib (HT-1080) was also the only one that lacked detectable Src kinase activity in this panel (Table 1). Moreover, suppression of cell migration and invasion was associated with inhibition of downstream Src signaling through FAK and p130CAS, proteins known to be involved in mediating these cellular processes (261-264). The IC50 values for inhibition of Src/FAK/p130CAS signaling as well as migration and invasion are all in the range of approximately 30 nM to 100 nM regardless of histological type (Table 1). Taken together, our findings suggest a model for the mechanism of dasatinib action in which blockade of Src and downstream signaling suppresses migration and invasion of sarcoma cells (Fig. 5C).

Significantly, dasatinib induces apoptosis in the majority of bone sarcoma cell lines, including OSA and EWS, but not in any of the soft tissue sarcoma cell lines in our
panel. Genetic inhibition of Src using siRNA also induced apoptosis in bone sarcoma cell lines that respond to dasatinib with apoptosis, but not in the only OSA cell line (MG-63) in which dasatinib did not induce apoptosis. Thus, dasatinib induces apoptosis in bone sarcoma cell lines dependent on Src kinase for survival. A major Src signaling pathway involved in preventing apoptosis in other cellular contexts is STAT3 (245, 265). While most of the sarcoma cell lines in this study harbor activated STAT3, with the sole exception of SK-ES-1, dasatinib did not inhibit STAT3 activation, indicating that this pathway is not involved in the dasatinib-mediated apoptosis response in sarcomas (our unpublished results). On the other hand, the IC₅₀ values for induction of apoptosis by dasatinib are in the same range required for blockade of FAK and p130CAS signaling in these cell lines. Because FAK and p130CAS have been implicated in tumor cell survival, in addition to cell migration and invasion, it is possible that these pathways are involved in dasatinib-mediated apoptosis in sarcoma cells.

It is notable that levels of Src activation do not correlate with IC₅₀ values of dasatinib responses in terms of cell migration, invasion or apoptosis (Table 1). This finding may be explained by the possibility that low levels of Src kinase activation are sufficient to induce these biological properties. Alternative explanations are the possibilities that other SFK members or unidentified targets are involved in the responses to dasatinib. Similar results have been observed for other molecular targeted-therapeutic agents, such as Iressa, where clinical response to this EGFR inhibitor is not correlated with levels of EGFR expression or activation (225). In the specific case of Iressa, EGFR mutations have been shown to influence response to Iressa; however, mutations in the c-Src gene are extremely rare in human cancers (230). Thus, selection of patients for
dasatinib treatment on the basis of Src expression or activation levels may not predict the optimal clinical responses. It remains to be determined whether any of the known genetic sub-types of sarcomas are more sensitive to dasatinib than others.

Earlier preclinical laboratory studies pointed to the promise of dasatinib in the treatment of Gleevec-resistant chronic myeloid leukemia, a prediction that has been borne out in clinical trials (266-272). On the basis of more recent preclinical laboratory studies, several human solid tumor sites have shown promise for clinical trials, including prostate, lung, pancreatic, and head and neck cancers (223-225). We have established that Src is activated in a wide variety of human sarcoma clinical specimens, including STS and bone sarcomas. Furthermore, our data demonstrate that dasatinib inhibits Src kinase and downstream signaling, leading to blockade of cell migration and invasion of sarcoma cell lines of diverse origins. In the subset of bone sarcomas, dasatinib also induces apoptosis. Taken together, our results suggest that dasatinib will provide clinical benefit to soft tissue and other sarcomas by preventing metastasis, which may be further augmented in bone sarcomas by induction of apoptosis.
Gene Expression Profile of Sarcoma Cell Lines Serves as Preliminary Signature Predictive of Response to Treatment with Dasatinib

It has recently been shown that unlike epithelial cancers, sarcomas are better defined by their molecular pathology rather than the organ of origin (1). Identified molecular alterations and cytogenetic analysis of specific subtypes of sarcomas have proven that previous classifications based on the site of the tumor are less important than the molecular phenotype of the tumor. Targeted therapies have aided in reaching this conclusion. Patients with specific molecular phenotypes have been shown to respond better to specific targeted therapies, as was the case with Gleevec and c-kit mutations in GISTs. These findings have had an important impact on the approaches in treating sarcomas. However, there are still many sarcomas subtypes with little to no improved treatment options.

Microarray analysis can help identify significant genes involved in sarcomagenesis and better classify tumor subtypes by molecular phenotype. Gene expression profiles (GEP) generated from microarray analysis have proven to successfully identify signatures that can predict prognosis and response to chemotherapies for breast cancer (273-276). Microarray analysis has recently provided insight into receptor tyrosine kinase expression patterns in sarcomas that may serve as potential targets for novel therapeutics. The examination of GISTs by microarray analysis lead to the identification of c-KIT and PDGFR as potential targets of therapy and
provided prognostic signatures that presented a biological basis for the differential responses exhibited to Gleevec. Furthermore, molecular signatures predicting response to therapy have been published for EWS and OSA (277). Consequently, the use of gene expression profiling by microarray analysis cannot only rapidly provide potential therapeutic targets, but may also serve as a screening mechanism used to predict response to a specific therapy, thereby preventing unnecessary patient exposure to various chemotherapeutics.

The second aim of this dissertation research was to identify a candidate molecular signature that predicts response to dasatinib by induction of apoptosis in human sarcoma cell lines. The response status of 12 cell lines was determined in aim one and was used to classify the cells into responsive and non-responsive categories. RNA was extracted from three consecutive passages of each cell line and purified for microarray analysis. A total of 36 Human Genome U133 Plus 2.0 Arrays were utilized for the initial analysis and six more for the second phase of this aim. Once a molecular signature was identified and validated, the accuracy of the signature to predict response in cell lines was tested using two new cell lines of unknown response status, to prevent bias. The same approach for RNA collection and purification was used for the test cell lines. Microarray analysis was performed independently of the molecular analysis and verification of response to dasatinib for each cell line. Once both analyses were completed, the results were compared. Amazingly, the results from both analyses were the identical; one cell line responded by induction of apoptosis to dasatinib, while the other did not. Therefore, a molecular signature that successfully predicts response to dasatinib by induction of apoptosis was identified in sarcoma cell lines.
Results

*Unsupervised clustering identified three main classes, with five subgroups in relation to cell line types*

Using an unsupervised hierarchical clustering approach, we tried to identify natural subclasses of cell lines as determined by gene expression profiles. We performed unsupervised clustering on a low-level filtered probe list using genes with median intensities of greater than or equal to 2000 to avoid using background noise in this analysis. The clustering results are shown in Figure 18A. Interestingly, three main classes were defined; the first included the EWS and RD cell lines, the second consisted of the STS and two OSA cell lines, while the third included three OSA cell lines. The first class is further sub-grouped by EWS and RD. Intriguingly, the A673 cell line, which was originally characterized as a RD, but later identified to possess an EWS translocation, was sub-grouped with the EWS arm. The second class consists of two sub-groups, one arm with two STS cell lines, the other arm with two OSA cell lines. Not surprising, the third class includes three cell lines which were derived from one of the cell lines in the sub-group.

A second hierarchical clustering approach was completed on the data set to determine if the cell lines could group according to response. This cluster was performed using a filtered probe of genes with median intensities greater than or equal to 2000 and had a significant (p-value ≤ 0.05) difference fold change between responders and non-responders. To calculate the fold change the median signal intensity of the non-responders was compared to the average signal intensity of each probe set and the cluster is shown in Figure 18B. This analysis provided two classes, one with all but one of the
non-responsive cell lines (A673) and the other with all but one responsive cell line (U2OS), suggesting that the cell lines can be separated into two distinct groups based on GEP.

**Figure 18.** Hierarchical clustering of sarcoma cell lines based on GEP. Microarray analysis was performed on 12 sarcoma cell lines in triplicate. The GEP of the cell lines was determined by calculating the average intensity per triplicate sample set and the fold change for each probe set using the median intensity for each probe set as the comparison group. The data were filtered for probe sets with ≥ 2000 intensity values, the remaining gene fold changes were transformed using Log2 and an unsupervised cluster was completed. A, shows the dendogram associated with this cluster. B, another hierarchical clustering analysis was performed, but the median intensity of the non-responders was used as the comparison group rather than the median of all samples. Data was further filtered for genes with fold changes that were significantly (p-value ≤ 0.05) different between responders and non-responders and had median signal intensities ≥ 2000.
A molecular signature distinguishes response to dasatinib as defined by induction of apoptosis

In order to stringently identify genes that might represent the molecular signature that predicts response to dasatinib, namely to select genes which have higher discrimination between the lists previously identified by the unsupervised analysis, we employed two different approaches of analysis. Our first approach was a SAM (statistical analysis of microarrays) analysis of the responsive versus the non-responsive cell lines as two groups. To prevent extracting a bone sarcoma specific signature, because those are the only cell lines that responded to dasatinib, we filtered out the bone sarcoma specific overlapping genes from the list. This provided a list of more than 1000 genes. Our second approach was to complete the standard MAS5 analysis. Once the signal intensities were determined for each probe the ratio for the maximum to minimum value for each probe set and each group was determined. Ratios less than 2.0 were included for additional analysis. This was completed to utilize the most consistent data for further analysis. Next, the fold change of responders was calculated. To complete this, the ratio of the average of each sample set for the responders to the median for all samples was calculated for each probe set. Probe sets with a significant fold change (p-value ≤0.05) were identified and selected to be compared to the probe sets which appeared on list generated using SAM. There were 26 probe sets that appeared on both lists, representing 22 different genes. Theses probe sets were selected as the molecular signature that predicts response to dasatinib (Figure 19A).

Probe sets for four genes: Histone H3, Fas associated factor-1 (FAF1), α-catenin and δ-catenin were further validated using qRT-PCR because more than one probe set
appeared on the molecular signature for these genes. The fold change values for these four genes are shown in Table 7 and Log₂ transformed fold changes are graphically depicted in Figure 19B and validated using qRT-PCR, the results of which are displayed in Table 8 and Log₂ fold changes are graphed in Figure 20. Histone H3 and FAF1 had higher fold expression, while α-catenin and δ-catenin had lower fold expression in the responsive cell lines.

The initial analysis of the 22 gene list provides the fold change of the responders compared to the median of all the cell lines analyzed and may dilute the precise fold change between responders and non-responders for this molecular signature. To determine the exact fold change between the two groups we also calculated the fold change using the ratio of the average responder signal for each cell line to the median non-responder signal intensity using the same probe set list. The heatmap generated for this analysis is shown in Figure 21A. This analysis provides a more pronounced fold change between the two groups for the molecular signature. Furthermore, the fold change for Histone H3, FAF1, α-catenin and δ-catenin are also shown in Table 9 and the Log₂ transformed fold changes are graphically depicted in Figure 21B and the trends of expression are upheld.
Figure 19. Molecular signature was generated using the median value for each probe set as comparison group. A, heatmap of 26 different probe sets representing 22 genes, the average was determined for each sample set and fold change was calculated using median of all samples for the comparison group. The probe sets on this list were significantly different among responders and non-responders (p-value ≤ 0.05) and appeared on the list for both analyses. B, graph expressing fold changes of four genes, Histone H3, FAF1, α-catenin and δ-catenin that appeared several times on the molecular signature. The fold changes calculated for A were transformed by Log2 and the values graphed to depict accurate fold changes between samples.
Table 7. Fold changes for key signature genes in sarcoma cell lines compared to median intensity

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Histone H3</th>
<th>Fas Associated Factor</th>
<th>α-Catenin</th>
<th>δ-Catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaOS-2</td>
<td>1.14 ± 0.21</td>
<td>1.62 ± 0.15</td>
<td>0.982 ± 0.13</td>
<td>0.640 ± 0.21</td>
</tr>
<tr>
<td>LM-2</td>
<td>1.30 ± 0.088</td>
<td>1.62 ± 0.089</td>
<td>0.925 ± 0.14</td>
<td>0.653 ± 0.024</td>
</tr>
<tr>
<td>LM-7</td>
<td>1.46 ± 0.11</td>
<td>1.49 ± 0.35</td>
<td>0.865 ± 0.10</td>
<td>0.478 ± 0.072</td>
</tr>
<tr>
<td>U 2 OS</td>
<td>1.09 ± 0.17</td>
<td>0.905 ± 0.14</td>
<td>0.909 ± 0.069</td>
<td>0.569 ± 0.089</td>
</tr>
<tr>
<td>MG-63</td>
<td>0.919 ± 0.11</td>
<td>0.947 ± 0.12</td>
<td>1.71 ± 0.19</td>
<td>1.27 ± 0.051</td>
</tr>
<tr>
<td>SK-ES-1</td>
<td>1.51 ± 0.12</td>
<td>0.965 ± 0.17</td>
<td>0.908 ± 0.080</td>
<td>0.732 ± 0.088</td>
</tr>
<tr>
<td>TC-71</td>
<td>1.33 ± 0.39</td>
<td>1.00 ± 0.12</td>
<td>0.684 ± 0.30</td>
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<td>SK-LMS</td>
<td>0.530 ± 0.03</td>
<td>0.823 ± 0.057</td>
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<tr>
<td>HT1080</td>
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<td>0.771 ± 0.057</td>
<td>1.26 ± 0.40</td>
<td>1.35 ± 0.45</td>
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<tr>
<td>A673</td>
<td>1.01 ± 0.13</td>
<td>0.678 ± 0.037</td>
<td>1.52 ± 0.13</td>
<td>1.48 ± 0.27</td>
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<tr>
<td>RD</td>
<td>0.680 ± 0.12</td>
<td>1.03 ± 0.078</td>
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<td>RD</td>
<td>18</td>
<td>0.690 ± 0.23</td>
<td>1.03 ± 0.25</td>
<td>0.913 ± 0.12</td>
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Figure 20. Quantitative RT-PCR validation of Histone H3, FAF1, α-catenin and δ-catenin gene expression. Bars represent Log2 fold changes for the selected genes. Positive fold change represents up-regulated, and negative fold change represents down-regulated in sarcoma cells.
Table 8. Quantitative RT-PCR expression of key signature genes in sarcoma cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Histone H3</th>
<th>Fas Associated Factor</th>
<th>α-Catenin</th>
<th>δ-Catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaOS-2</td>
<td>0.865 ± 0.016</td>
<td>1.04 ± 0.14</td>
<td>1.10 ± 0.13</td>
<td>1.10 ± 0.0058</td>
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<td>LM-2</td>
<td>1.45 ± 0.010</td>
<td>2.13 ± 0.12</td>
<td>1.08 ± 0.19</td>
<td>1.14 ± 0.0070</td>
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<tr>
<td>LM-7</td>
<td>3.43 ± 0.025</td>
<td>4.17 ± 0.18</td>
<td>1.13 ± 0.062</td>
<td>1.04 ± 0.025</td>
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<tr>
<td>U2OS</td>
<td>0.227 ± 0.018</td>
<td>0.187 ± 0.18</td>
<td>1.13 ± 0.037</td>
<td>1.03 ± 0.036</td>
</tr>
<tr>
<td>MG-63</td>
<td>0.500 ± 0.0089</td>
<td>0.483 ± 0.18</td>
<td>1.13 ± 0.10</td>
<td>1.07 ± 0.015</td>
</tr>
<tr>
<td>SK-ES-1</td>
<td>1.33 ± 0.0049</td>
<td>0.637 ± 0.20</td>
<td>1.15 ± 0.015</td>
<td>1.01 ± 0.016</td>
</tr>
<tr>
<td>TC-71</td>
<td>2.62 ± 0.010</td>
<td>1.21 ± 0.082</td>
<td>1.06 ± 0.17</td>
<td>1.13 ± 0.020</td>
</tr>
<tr>
<td>SK-LMS</td>
<td>2.95 ± 0.0077</td>
<td>4.29 ± 0.15</td>
<td>1.11 ± 0.15</td>
<td>1.11 ± 0.014</td>
</tr>
<tr>
<td>HT1080</td>
<td>0.160 ± 0.0043</td>
<td>0.334 ± 0.19</td>
<td>1.14 ± 0.12</td>
<td>1.08 ± 0.035</td>
</tr>
<tr>
<td>A673</td>
<td>0.812 ± 0.0073</td>
<td>0.551 ± 0.19</td>
<td>1.14 ± 0.10</td>
<td>1.07 ± 0.016</td>
</tr>
<tr>
<td>RD</td>
<td>0.337 ± 0.020</td>
<td>0.559 ± 0.23</td>
<td>1.17 ± 0.013</td>
<td>1.01 ± 0.054</td>
</tr>
<tr>
<td>RD</td>
<td>18</td>
<td>0.818 ± 0.087</td>
<td>1.23 ± 0.011</td>
<td>1.01 ± 0.28</td>
</tr>
</tbody>
</table>
Figure 21. Molecular signature generated using the median value for each probe set in the non-responders as comparison group. A, heatmap of 26 different probe sets representing 22 genes, the average was determined for each sample set and fold change was calculated using median of the non-responders as comparison group for each probe set. The probe sets on this list were significantly different among responders and non-responders (p-value ≤ 0.05) and appeared on the list for both analyses. B, graph expressing fold changes of Histone H3, FAF1, α-catenin and δ-catenin. The fold changes calculated for A were transformed by Log2 and the values graphed to depict accurate fold changes between samples.
Table 9. Fold Changes for key signature genes in sarcoma cells compared to median intensity of non-responders

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Histone H3</th>
<th>Fas Associated Factor</th>
<th>α-Catenin</th>
<th>δ-Catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaOS-2</td>
<td>1.63 ± 0.31</td>
<td>1.95 ± 0.18</td>
<td>0.854 ± 0.11</td>
<td>0.482 ± 0.16</td>
</tr>
<tr>
<td>LM-2</td>
<td>1.86 ± 0.12</td>
<td>1.96 ± 0.10</td>
<td>0.804 ± 0.13</td>
<td>0.493 ± 0.018</td>
</tr>
<tr>
<td>LM-7</td>
<td>2.09 ± 0.15</td>
<td>1.81 ± 0.42</td>
<td>0.752 ± 0.090</td>
<td>0.360 ± 0.054</td>
</tr>
<tr>
<td>U 2 OS</td>
<td>1.56 ± 0.24</td>
<td>1.10 ± 0.16</td>
<td>0.792 ± 0.061</td>
<td>0.429 ± 0.067</td>
</tr>
<tr>
<td>MG-63</td>
<td>1.311 ± 0.15</td>
<td>1.15 ± 0.15</td>
<td>1.49 ± 0.17</td>
<td>0.955 ± 0.038</td>
</tr>
<tr>
<td>SK-ES-1</td>
<td>2.16 ± 0.18</td>
<td>1.18 ± 0.21</td>
<td>0.789 ± 0.069</td>
<td>0.552 ± 0.067</td>
</tr>
<tr>
<td>TC-71</td>
<td>1.91 ± 0.56</td>
<td>1.20 ± 0.14</td>
<td>0.594 ± 0.26</td>
<td>0.756 ± 0.0040</td>
</tr>
<tr>
<td>SK-LMS</td>
<td>0.756 ± 0.037</td>
<td>0.993 ± 0.069</td>
<td>1.08 ± 0.18</td>
<td>1.33 ± 0.19</td>
</tr>
<tr>
<td>HT1080</td>
<td>0.915 ± 0.15</td>
<td>0.931 ± 0.067</td>
<td>1.10 ± 0.35</td>
<td>1.02 ± 0.34</td>
</tr>
<tr>
<td>A673</td>
<td>1.44 ± 0.18</td>
<td>0.820 ± 0.046</td>
<td>1.32 ± 0.12</td>
<td>1.12 ± 0.21</td>
</tr>
<tr>
<td>RD</td>
<td>0.970 ± 0.17</td>
<td>1.26 ± 0.090</td>
<td>0.877 ± 0.13</td>
<td>0.971 ± 0.13</td>
</tr>
<tr>
<td>RD</td>
<td>18</td>
<td>0.984 ± 0.32</td>
<td>1.25 ± 0.30</td>
<td>0.793 ± 0.10</td>
</tr>
</tbody>
</table>
Identification of two probe sets with greater fold changes may provide further insight into the prediction of response

To identify possible probe sets that may convey a greater fold change between response groups, we altered our search criteria by increasing the ratio of the maximum to minimum ratio value for each probe set to 10 and searched for probe sets with \( \geq 5.0 \) or \( \leq -5.0 \) fold change between groups. Of the genes provided from this search two probe sets with interesting potential were followed-up; Ephrin-A1, the ligand of a known target of dasatinib, EphA2 receptor, and Dapper, an antagonist of catenin, both of which have recently been shown to play unique roles in the malignant phenotype. When clustered based on the expression of these two genes, the heatmap generated from this search shows that the responders and non-responders completely cluster apart on separate arms when the fold change is calculated using the median probe set intensity as comparison or the median non-responder probe set intensity (Figures 22A and 23A). In addition, a graphic depiction of the Log2 transformed fold changes for these two genes shows greater expression of both Ephrin-A1 and Dapper in the responsive cell lines as compared to the non-responsive cell lines using either median comparison group (Figures 22B and 23B). The values of relative fold change are also shown on Tables 10 and 11 for both analyses.

Quantitative RT-PCR was completed on the cell lines for \textit{ephrin-A1} and \textit{dapper} to validate the microarray results. Figure 24 shows the graphic expression of these two genes compared to the median expression of all samples, and Table 11 shows the absolute values of the average expression for \textit{ephrin-A1} and \textit{dapper} in the cell lines generated from the analysis. The validation concludes that the microarrays accurately revealed an increased expression of Ephrin-A1 and Dapper in the responsive cell lines.
Figure 22. Expression of Ephrin-A1 and Dapper in sarcoma cell lines using the median of all samples as comparison group.  

A, heatmap of Ephrin-A1 and Dapper fold changes in 12 sarcoma cell lines. The average was determined for each sample set and the fold change was calculated by using the median of all samples as the comparison group for each probe set.  

B, graph of Ephrin-A1 and Dapper fold change between sample sets. The fold changes were calculated by transforming the fold changes calculated in A by Log₂.
Figure 23. Expression of Ephrin-A1 and Dapper in sarcoma cell lines using the median of the non-responders as comparison group. **A**, heatmap of Ephrin-A1 and Dapper fold changes in 12 sarcoma cell lines. The average was determined for each sample set and the fold change was calculated by using the median of the non-responders as the comparison group for each probe set. **B**, graph of Ephrin-A1 and Dapper fold change between sample sets. The fold changes were calculated by transforming the fold changes calculated in **A** by Log2.
Table 10. Fold changes for ephrin-a1 and dapper in sarcoma cell lines compared to median intensity

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Ephrin-A1</th>
<th>Dapper</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaOS-2</td>
<td>3.71 ± 0.66</td>
<td>5.39 ± 1.5</td>
</tr>
<tr>
<td>LM-2</td>
<td>3.60 ± 0.93</td>
<td>3.54 ± 0.44</td>
</tr>
<tr>
<td>LM-7</td>
<td>2.94 ± 0.87</td>
<td>5.63 ± 1.1</td>
</tr>
<tr>
<td>U 2 OS</td>
<td>1.13 ± 0.82</td>
<td>3.02 ± 0.86</td>
</tr>
<tr>
<td>MG-63</td>
<td>0.354 ± 0.13</td>
<td>0.252 ± 0.25</td>
</tr>
<tr>
<td>SK-ES-1</td>
<td>3.26 ± 0.78</td>
<td>1.06 ± 0.21</td>
</tr>
<tr>
<td>TC-71</td>
<td>1.27 ± 0.78</td>
<td>0.914 ± 0.23</td>
</tr>
<tr>
<td>SK-LMS</td>
<td>0.136 ± 0.029</td>
<td>0.374 ± 0.047</td>
</tr>
<tr>
<td>HT1080</td>
<td>0.159 ± 0.17</td>
<td>0.328 ± 0.092</td>
</tr>
<tr>
<td>A673</td>
<td>0.055 ± 0.17</td>
<td>0.954 ± 0.18</td>
</tr>
<tr>
<td>RD</td>
<td>0.237 ± 0.31</td>
<td>0.440 ± 0.30</td>
</tr>
<tr>
<td>RD</td>
<td>18</td>
<td>0.388 ± 0.078</td>
</tr>
</tbody>
</table>
Table 11. Microarray fold changes for ephrin-a1 and dapper in sarcoma cell lines compared to median intensity of non-responders

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Ephrin-A1</th>
<th></th>
<th></th>
<th>Dapper</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SaOS-2</td>
<td>10.3 ± 1.8</td>
<td>14.9 ± 4.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM-2</td>
<td>10.0 ± 2.6</td>
<td>9.77 ± 1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM-7</td>
<td>8.19 ± 2.4</td>
<td>15.5 ± 3.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U 2 OS</td>
<td>3.16 ± 2.3</td>
<td>8.33 ± 2.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG-63</td>
<td>1.38 ± 0.35</td>
<td>0.921 ± 0.69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK-ES-1</td>
<td>9.10 ± 2.1</td>
<td>2.92 ± 0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC-71</td>
<td>3.55 ± 2.2</td>
<td>2.52 ± 0.62</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SK-LMS</td>
<td>4.67 ± 0.081</td>
<td>2.12 ± 0.13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT0180</td>
<td>0.983 ± 0.47</td>
<td>0.790 ± 0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A673</td>
<td>0.705 ± 0.48</td>
<td>3.05 ± 0.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>1.52 ± 0.88</td>
<td>1.02 ± 0.83</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>18</td>
<td>1.10 ± 0.22</td>
<td>0.772 ± 0.45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 24. Quantitative RT-PCR validations of Ephrin-A1 and Dapper genes. Bars represent fold changes for the selected genes. Positive fold change represents up-regulated, and negative fold change represents down-regulated in sarcoma cells.
Table 12. Quantitative RT-PCR values for ephrin-a1 and dapper in sarcoma cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Ephrin-A1</th>
<th>Dapper</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaOS-2</td>
<td>1.74 ± 0.11</td>
<td>1.64 ± 0.16</td>
</tr>
<tr>
<td>LM-2</td>
<td>4.44 ± 0.28</td>
<td>2.90 ± 0.19</td>
</tr>
<tr>
<td>LM-7</td>
<td>5.58 ± 0.40</td>
<td>11.7 ± 0.31</td>
</tr>
<tr>
<td>U 2 OS</td>
<td>1.30 ± 0.16</td>
<td>1.76 ± 0.11</td>
</tr>
<tr>
<td>MG-63</td>
<td>0.197 ± 0.028</td>
<td>0.228 ± 0.017</td>
</tr>
<tr>
<td>SK-ES-1</td>
<td>3.21 ± 0.12</td>
<td>0.603 ± 0.057</td>
</tr>
<tr>
<td>TC-71</td>
<td>2.25 ± 0.13</td>
<td>1.68 ± 0.14</td>
</tr>
<tr>
<td>SK-LMS</td>
<td>0.474 ± 0.018</td>
<td>1.05 ± 0.022</td>
</tr>
<tr>
<td>HT1080</td>
<td>0.212 ± 0.028</td>
<td>0.156 ± 0.011</td>
</tr>
<tr>
<td>A673</td>
<td>0.603 ± 0.075</td>
<td>0.954 ± 0.11</td>
</tr>
<tr>
<td>RD</td>
<td>0.406 ± 0.077</td>
<td>0.061 ± 0.019</td>
</tr>
<tr>
<td>RD</td>
<td>18</td>
<td>0.700 ± 0.12</td>
</tr>
</tbody>
</table>
Testing the molecular signature with cell lines of unknown response reveals that the molecular signature can accurately predict response to dasatinib in cell lines

To test the molecular signature of response, two new cell lines of unknown response status were acquired. Microarray and molecular analysis were independently performed and the response status generated from the two analyses were compared. To determine response status of the new cell lines the fold change of each probe set was calculated by taking the ratio the average intensity for each probe set in the new cell lines to the median intensity of the original dataset and compared the fold change to the molecular signature extracted from the original group of cell lines. Upon further examination of the gene expression profile of the two cell lines and comparison to the molecular signature, HOS was characterized as a responsive cell line and SW1353 was characterized as a non-responsive cell line. Each cell line also clustered with its respective group as is observed in Figure 25A.

In addition, the expression of Histone H3, FAF1 and δ-catelin were significantly different in each of the cell lines. Histone H3 and FAF1 had greater expression, while δ-catelin had a decreased expression in the HOS cell line (Figure 25B, Table 13). The expression of these three genes correlates with the expected expression patterns according to the predictive molecular signature. However, when q-RT-PCR was performed to validate the expression of these genes in the HOS and SW1353 cell lines, Histone H3 and FAF1 were significantly upregulated in the HOS cell line compared to the SW1353 cell line. The expression of δ-catelin was not significantly different, although it was upregulated in SW1353 as compared to HOS. While, the expression α-
catenin was also significantly different in the two cell lines, the opposite expression pattern was observed than expected.

To validate the characterization of the cell lines dose response to dasatinib was completed in both cell lines at 6, 24 and 72 h. Dasatinib inhibited Src activation, signaling and cellular migration in both cell lines (Figure 26A-E). However, HOS was the more sensitive of the two cell lines, in that a lower dose of dasatinib was required to inhibit Src activation. Furthermore, dasatinib also induced apoptosis in the HOS cell line and not the SW1353 cell line (Figure 27A and B). These results confirm the characterization of the cell lines by microarray analysis. Thus, these data successfully validated the use of our signature in the ability to predict the response of human sarcoma cell lines to dasatinib.

On the other hand, Ephrin-A1 and Dapper expression did not correlate with the expected expression pattern in accordance with response. HOS was observed as having significantly lower expression of both genes as compared to SW1353 (Figure 28A, C and 29A, C). Furthermore, when clustered based on the expression of these two genes, HOS and SW1353 clustered with the opposite response groups (Figures 28B and 28B). The values of Ephrin-A1 and Dapper can be further assessed in Tables 15 and 16. Several possibilities could explain the lack of validation of Ephrin-A1 and Dapper as predictive genes and will be further articulated in the discussion.
Figure 25. Molecular signature that predicts response to dasatinib generated using the median value for each probe set as comparison group. A, the same molecular signature was used to create this heatmap as for figure 20A. The only difference in this heatmap is the presence of two new cell lines, HOS and SW1353. The average was determined for each sample set and fold change was calculated using median of all samples for the comparison group to calculate the fold changes utilized for this heatmap. Samples were clustered based on the GEP of the 26 probe set list. B, graph expressing fold changes of Histone H3, FAF1, α-catenin and δ-catenin in HOS and SW1353 cells. The fold changes calculated for A were transformed by Log2 and the values graphed to depict accurate fold changes between samples. C, graph expressing the relative qRT-PCR fold changes of Histone H3, FAF1, α-catenin and δ-catenin in HOS and SW1353 cells using the median expression for comparison. (* p-value \(\leq 0.01\), ** p-value \(\leq 0.0001\))
Table 13. Fold changes key signature genes in test cells compared to median intensity

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Histone H3</th>
<th>Fas Associated Factor</th>
<th>α-Catenin</th>
<th>δ-Catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOS</td>
<td>1.16 ± 0.14</td>
<td>0.907 ± 0.072</td>
<td>0.93 ± 0.12</td>
<td>1.04 ± 0.12</td>
</tr>
<tr>
<td>SW1353</td>
<td>1.05 ± 0.17</td>
<td>0.508 ± 0.040</td>
<td>0.88 ± 0.086</td>
<td>1.20 ± 0.063</td>
</tr>
</tbody>
</table>

Table 14. Relative qRT-PCR fold changes key signature genes in test cells compared to median intensity

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Histone H3</th>
<th>Fas Associated Factor</th>
<th>α-Catenin</th>
<th>δ-Catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOS</td>
<td>3.05 ± 0.035</td>
<td>2.46 ± 0.11</td>
<td>2.51 ± 0.12</td>
<td>1.21 ± 0.045</td>
</tr>
<tr>
<td>SW1353</td>
<td>1.91 ± 0.017</td>
<td>1.21 ± 0.15</td>
<td>1.77 ± 0.085</td>
<td>1.62 ± 0.0023</td>
</tr>
</tbody>
</table>
Figure 26. Dasatinib inhibits Src activation and signaling in HOS and SW1353 cells. A and C, HOS and SW1353 cells were treated with dasatinib in a dose-dependent manner for 6 h. Cell-free extracts were immunoblotted with antibodies specific to p-Src (Y419) and total Src. B and D, dasatinib specifically blocks tyrosyl phosphorylation of FAK (Y576/577, Y925) and 130CAS (Y410), but not FAK Y397. HOS and SW1353 cells were treated with dasatinib for 6 h in a dose-dependent manner. Cell-free extracts were immunoblotted with antibodies specific to p-FAK (Y397, Y576/577, Y925), total FAK, p130CAS and p-p130CAS (Y410). β-actin was blotted for as a loading control in all experiments. Dasatinib inhibits cell motility and invasion. E, wound healing assays were performed on HOS and SW1353 to determine the effects of dasatinib on inhibiting cell migration as completed previously. Cell migration was visualized at 10x magnification by light microscopy and photographed with a digital camera. The width of voided area versus dasatinib dose concentration was graphed to express the degree of inhibition of cell migration. The number of pixels within the denuded area were the units used to demonstrate inhibition of cell migration induced by dasatinib (*p<0.001, n=3).
Figure 27. Dasatinib induces apoptosis in HOS but not SW1353 sarcoma cells. A, dasatinib induces apoptosis in a dose-dependent manner in HOS cell only. Cells were treated with dasatinib for 72 h with escalating doses. Cell-free extracts were immunoblotted with antibodies specific to XIAP and PARP. β-actin was blotted for as a loading control in all experiments.
Figure 28. Expression of Ephrin-A1 and Dapper in HOS and SW1353 cells using the median of all samples as comparison group. A, heatmap of Ephrin-A1 and Dapper fold changes in HOS and SW1353 cells. The average was determined for each sample set and the fold change was calculated by using the median of all samples as the comparison group for each probe set. B, integration of HOS and SW1353 into the original heatmap of Ephrin-A1 and Dapper expression. C, graph of Ephrin-A1 and Dapper fold changes in HOS and SW1353. The fold changes were calculated by transforming the fold changes calculated in A by Log2. (* p-value ≤ 0.01)
Figure 29. Expression of Ephrin-A1 and Dapper in HOS and SW1353 cells using the median of the non-responders comparison group. A, heatmap of Ephrin-A1 and Dapper fold changes in HOS and SW1353 cells. The average was determined for each sample set and the fold change was calculated by using the median of the non-responders as the comparison group for each probe set. B, integration of HOS and SW1353 into the original heatmap of Ephrin-A1 and Dapper expression. C, graph of Ephrin-A1 and Dapper fold changes in HOS and SW1353. The fold changes were calculated by transforming the fold changes calculated in A by Log₂. (* p-value ≤ 0.001)
Table 15. Fold changes for ephrin-a1 and dapper in test cells compared to median intensity

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Ephrin A1</th>
<th>Dapper</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOS</td>
<td>0.526 ± 0.10</td>
<td>1.03 ± 0.23</td>
</tr>
<tr>
<td>SW1353</td>
<td>0.926 ± 0.25</td>
<td>10.1 ± 1.2</td>
</tr>
</tbody>
</table>

Table 16. Microarray fold changes for ephrin-a1 and dapper in test cells compared to median intensity of non-responders

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Ephrin A1</th>
<th>Dapper</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOS</td>
<td>0.534 ± 0.30</td>
<td>1.48 ± 0.31</td>
</tr>
<tr>
<td>SW1353</td>
<td>1.33 ± 0.38</td>
<td>4.80 ± 0.17</td>
</tr>
</tbody>
</table>

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Discussion

Microarray analysis of gene expression profiles in sarcomas is a valuable technique that provides a comprehensive survey of activated molecular pathways and has been shown to successfully identify potential therapeutic targets. In this aim, we attempted to identify a molecular signature that will predict response to dasatinib by induction of apoptosis in sarcoma cell lines. As a model, we initially compared the GEP of 12 sarcoma cell lines characterized by response to dasatinib. The unsupervised analysis of GEP data clearly separated the cell lines by tumor type (Figure 19A). Using statistical filtering to identify genes significantly different between the responders and non-responders, the GEP data analyzed created two distinct classes, with all but one cell line from each group clustering within their corresponding classes (Figure 19B). Then, using two robust statistical filtering procedures, a 22 gene signature with a high discrimination power among the resistant and non-resistant groups was identified (Figure 20A and 21A). Interestingly, many of the genes present in the signature are found within close proximity on chromosome one, particularly Histone H3 and FAF1 are located on chromosome 1q21, which is an area that has been shown to be amplified in OSA. Of the 22 genes in this signature four genes were selected for further analysis because multiple probe sets for these genes appeared on the signature with consistent fold changes between classes; Histone H3, FAF1, α-catenin and δ-catenin. Histone H3 and FAF1 were upregulated, while α-catenin and δ-catenin were downregulated in the responsive group as a whole (Figure 19B and 21B; Tables 7 and 9) and these findings were further validated by qRT-PCR (Table 8, Figure 20).
Two other probe sets were identified as potential predictors of response using a variation of our initial analysis; Ephrin-A1 and Dapper. Ephrin-A1, a known target of dasatinib, and Dapper, an antagonist of catenins, were both upregulated in the responsive cell lines (Figures 22A and 23A) and this upregulation was validated by qRT-PCR (Figure 24, Table 12). Upon identification of an RNA signature, our next goal was to test whether this signature can successfully characterize cell lines into their respective groups based on response to dasatinib. To test the signature we acquired two new cell lines; HOS, an OSA, and SW1353, a CS cell line of unknown response to dasatinib.

Microarray analysis was performed independently as the molecular biology assays were performed to determine the response status of the two cell lines. The results of both analyses were compared once the results for each were determined. The results of both analyses agreed; the microarray analysis grouped the HOS with the responders and SW1353 with the non-responders based on the expression of the molecular signature (Figure 25A). Furthermore, Histone H3, FAF1 and δ-catenin expression were significantly different in the two cell lines and corresponded with the expression patterns of their respective classes. The molecular biology analysis determined that HOS responded to dasatinib by induction of apoptosis, while SW1353 merely responded by inhibition of Src signaling and migration (Figure 26A-E and Figure 27A-B).

Alternatively, prediction of response based on the expression of Ephrin-A1 and Dapper did not successfully classify the cell lines into the correct classes (Figures 28 and 29; Table 15 and 16). In fact, the cell lines were characterized into the complete opposite groups based on the expression of these two genes. One possible explanation for this finding could be that only two cell lines were used to test the predictive characterization
of these genes, perhaps if more cell lines were utilized for the validation of Ephrin-A1 and Dapper their ability to be used in predicting response would be more promising. Another explanation may be that these two genes alone, are not sufficient enough to predict response, but when applied together within the context of the signature may possess more significance.

Nevertheless, the class prediction analysis performed using these 26 probe sets as a predictor gene list for two cell lines of unknown response successfully classified the cell lines with their respective response groups. These findings lend evidence to suggest that this molecular signature can to predict response to dasatinib in cell lines and aid in identifying cell lines that required Src activity for survival. To further validate this signature, our next aim was to establish whether the signature was present in primary human sarcomas. Should the signature be expressed in sarcomas, future clinical trials with dasatinib could include a component to test the validity of the molecular signature in predicting response in sarcoma patients.
Validation of Gene Expression Profile in Primary Human Sarcomas

The primary objective of this aim is to determine whether the molecular signature that predicts response to dasatinib by induction of apoptosis in cell lines is expressed in human sarcomas. Validating the expression of the signature predictive of response to Src inhibition may lay the foundation for designing future clinical trials that will help tailor design treatment options for sarcoma patients. In addition to designing trials, the results gained from this research will be useful for the impending clinical trial to evaluate dasatinib as a potential treatment option for sarcomas. If present, the molecular signature could be tested using samples gathered in the future trial and validated as a potential predicative signature. In addition, the results of this study will contribute to our understanding of the response to treatment of tumors where histological evaluation and ancillary studies have proven to be insufficient predictors.

In the present study, we applied microarray gene expression profiling to establish the presence of a candidate molecular signature, from the cell lines that predicts response to dasatinib, in human sarcomas. Our goal was to obtain untreated, primary human sarcoma specimens collected under a general tissue banking consent protocol. The tumor histologies utilized for this aim corresponded to the tumor types represented in the cell line component of the study, including OSA, LMS, FBS and RD. Unfortunately, there was no RNA available for the EWS that were collected under this protocol. To compensate for the lack of samples, sarcomas representing other histologies were also
utilized for these investigations. GEPs were established for each specimen and compared to the molecular signature extracted from the cell lines. The comparison was completed to determine whether components of the cell line derived molecular signature were present in human sarcomas.

To our astonishment, 10 or the 22 sarcomas used for this study expressed significant components of the molecular signature from the cell lines. The expression of the signature also provided a theoretical assignment of potential response to dasatinib. Furthermore, the expression of the molecular signature was significant enough to cluster many of the tumors based on their GEPs into their theoretical response groups. These data lend evidence to suggest that at least components of the cell line signature are expressed in sarcomas. These results are encouraging and support the implementation of further investigation into the expression and predictability of this signature to predict response in primary sarcomas.
Results

Unsupervised clustering identified diversity among human sarcoma specimens

Using an unsupervised hierarchical clustering approach, we tried to identify natural subclasses of tumor specimens as determined by gene expression profiles. We performed the unsupervised clustering on an unfiltered probe list and have shown that sarcomas are not easily grouped into tumor subclasses based on gene expression alone (Figure 30A). In fact, the only subgroup that clustered together was the two OSA specimens. The remaining sarcomas clustered with apparently no preference for tumor subgroup.

Identification of the cell line molecular signature in human sarcomas

Our next goal was to determine whether components of the 26 gene molecular signature are present in human sarcoma specimens. To complete this we compared the intensity of each probe set for the tumors, calculated fold change by comparing the intensity for each probe set to the median intensity of the dataset and compared the fold change to the molecular signature extracted from the cell lines. Of the 22 tumors analyzed, 10 were characterized as potential responders and 12 were characterized as potential non-responders based on the fold changes of the genes that comprise the molecular signature that predicts response in cell lines (Table 16). A breakdown of the percentage responders and non-responders by tumor subgroups is illustrated in Figure 30B. Figure 30A identifies the potential responsive tumors with red stars above the sample label. Clearly the tumors do not cluster by potential response to dasatinib when unsupervised and unfiltered.
Figure 30. Hierarchical clustering of primary sarcoma specimens. The GEP of the tumors was determined by calculating fold change for each probe set, using the average intensity of each probe set across samples as the comparison group. An unsupervised cluster was performed to determine inherent similarities between tumors, A. Sarcomas categorized as potential responders are indicated with *. B, breakdown of tumors categorized as potential responders and non-responders by molecular signature according to sarcoma type.
Table 17. Classification of sarcomas using predictive signature from cell lines

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Responder</th>
<th>Non-Responder</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS T98</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>CS T2355</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>CS T2744</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>CS T3109</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>FBS T3946</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>FBS T4436</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>FBS T6390</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>LMS T174</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>LMS T191</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>LMS T294</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>LMS T374</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>LMS T466</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>LMS T3863</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>LPS T2500</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>MFH T1913</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>MFH T5087</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>MFS T6251</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>OSA T1898</td>
<td>R</td>
<td></td>
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<tr>
<td>OSA T6357</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>RD T395</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>SCS T548</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>SCS T7431</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10</strong></td>
<td><strong>12</strong></td>
</tr>
</tbody>
</table>
Figure 31. Heatmap of molecular signature expression in sarcomas. Fold changes for the molecular signature were calculated by using the average intensity for each probe set as the comparison group. A, heatmap of unclustered tumors arranged by response category. B, heatmap of tumors clustered based on the expression of the molecular signature used to define theoretical response status. Dendogram at top of heatmap shows the relationship of tumors based on the GEP of the molecular signature. Sarcomas categorized as potential responders are indicated with a *. 
Molecular signature that predicts response to dasatinib in cell lines can be used to group tumors by potential response

An unclustered heatmap of the molecular signature expression in the tumors arranged by potential response does not present a clear separation by expression between the two groups (Figure 31A). Furthermore, when clustered based on the GEP of the molecular signature, the tumors do not completely cluster into their respective groups (Figure 31B). However, there is some promise to a trend of consistency in the clustering, in that some of the tumors did cluster into their respective groups; 6 out of 10 for the responders clustered together and 8 out of 12 of the non-responders. There are several possibilities to explain this inconsistency and will be addressed in the discussion.

A heatmap was also prepared for the tumor expression of the four genes validated from the predictive molecular signature, Histone H3, FAF1 α-catenin and δ-catenin, When clustered, again the tumors classified as potential responders and non-responders did not cluster in two separate groups. Although, 70% of responsive tumors clustered together and 50% of the non-responsive tumors clustered together (Figure 31A). The expression patterns of Histone H3, FAF1 α-catenin and δ-catenin are also inconsistent when are pulled from the GEP and compared to the cell line signature (Figure 32A-B, Table 17). Again, there is a trend of recurrent expression patterns between responsive verses the non-responsive groups, although it is not uniform across all samples.
Figure 32. Expression of Histone H3, FAF1, α-Catenin and δ-Catenin in sarcomas. A, heatmap of Histone H3, FAF1, α-catenin and δ-catenin extracted from molecular signature and clustered based on GEP of these four genes. B, graphic depiction of fold changes associated with Histone H3, FAF1, α-catenin and δ-catenin in tumors. Sarcomas categorized as potential responders are indicated with a *.
Table 18. Fold changes for key signature genes in sarcomas

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Histone H3</th>
<th>Fas Associated Factor</th>
<th>α Catenin</th>
<th>δ Catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS T98</td>
<td>1.22</td>
<td>1.17</td>
<td>0.969</td>
<td>1.17</td>
</tr>
<tr>
<td>CS T3109</td>
<td>0.919</td>
<td>0.753</td>
<td>0.875</td>
<td>0.834</td>
</tr>
<tr>
<td>LMS T174</td>
<td>1.405</td>
<td>1.15</td>
<td>0.861</td>
<td>0.718</td>
</tr>
<tr>
<td>LMS T191</td>
<td>1.24</td>
<td>0.721</td>
<td>1.49</td>
<td>1.59</td>
</tr>
<tr>
<td>LMS T294</td>
<td>1.18</td>
<td>0.804</td>
<td>1.09</td>
<td>1.56</td>
</tr>
<tr>
<td>LMS T466</td>
<td>1.57</td>
<td>1.31</td>
<td>0.996</td>
<td>1.43</td>
</tr>
<tr>
<td>LPS T2500</td>
<td>0.901</td>
<td>0.818</td>
<td>1.06</td>
<td>1.25</td>
</tr>
<tr>
<td>OSA T1898</td>
<td>1.23</td>
<td>0.712</td>
<td>1.03</td>
<td>0.965</td>
</tr>
<tr>
<td>OSA T6357</td>
<td>1.42</td>
<td>1.24</td>
<td>1.13</td>
<td>0.781</td>
</tr>
<tr>
<td>SCS T7431</td>
<td>0.769</td>
<td>0.798</td>
<td>1.84</td>
<td>1.43</td>
</tr>
<tr>
<td>CS T2355</td>
<td>1.12</td>
<td>0.661</td>
<td>1.18</td>
<td>0.793</td>
</tr>
<tr>
<td>CS T2744</td>
<td>0.611</td>
<td>1.105</td>
<td>0.973</td>
<td>0.902</td>
</tr>
<tr>
<td>FBS T3946</td>
<td>0.881</td>
<td>0.940</td>
<td>0.964</td>
<td>1.04</td>
</tr>
<tr>
<td>FBS T4436</td>
<td>0.947</td>
<td>1.07</td>
<td>1.40</td>
<td>1.20</td>
</tr>
<tr>
<td>FBS T6390</td>
<td>0.859</td>
<td>0.955</td>
<td>0.856</td>
<td>0.693</td>
</tr>
<tr>
<td>LMS T374</td>
<td>1.23</td>
<td>1.73</td>
<td>1.83</td>
<td>1.66</td>
</tr>
<tr>
<td>LMS T3863</td>
<td>1.04</td>
<td>1.90</td>
<td>0.664</td>
<td>0.898</td>
</tr>
<tr>
<td>MFH T1913</td>
<td>0.732</td>
<td>1.87</td>
<td>0.983</td>
<td>0.894</td>
</tr>
<tr>
<td>MFH T5087</td>
<td>0.832</td>
<td>0.716</td>
<td>0.963</td>
<td>1.06</td>
</tr>
<tr>
<td>MFS T6251</td>
<td>0.611</td>
<td>1.13</td>
<td>0.674</td>
<td>0.686</td>
</tr>
<tr>
<td>RD T395</td>
<td>0.789</td>
<td>1.25</td>
<td>1.11</td>
<td>0.901</td>
</tr>
<tr>
<td>SCS T548</td>
<td>1.14</td>
<td>1.24</td>
<td>0.740</td>
<td>1.01</td>
</tr>
</tbody>
</table>
Analysis of two probe sets with greater fold changes may provide further insight into the prediction of response in tumors

The expression of Ephrin-A1 and Dapper were also analyzed in the tumors specimens. A heatmap with hierarchical clustering produced three classes of tumors based on Ephrin-A1 and Dapper gene expression. While none of the three groups contained all of one response group, the clustering is promising. One group contained one responder and three non-responders, the second group had seven responders and two non-responders, while the third group included two responders and seven non-responders (Figure 33A). The fold change and expression patterns of Ephrin-A1 and Dapper correlate more with the cell lines, in that more of the tumors that express the potentially responsive signature had greater fold changes for Ephrin-A1 and Dapper as compared to the potentially non-responsive tumors as a whole (Figure 33B, Table 18).
**Figure 33.** Expression of Ephrin-A1 and Dapper in sarcomas. The fold changes of Ephrin-A1 and Dapper were calculated using the median intensity of all samples as the comparison group. *A*, Hierarchical clustering was performed based on the GEP of Ephrin-A1 and Dapper in sarcomas. *B*, graphic depiction of Ephrin-A1 and Dapper fold changes between primary sarcoma specimens as compared to the median intensity for each probe set. Sarcomas categorized as potential responders are indicated with a *. 
**Table 19.** Fold changes for ephrin-a1 and dapper in sarcomas

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Ephrin-A1</th>
<th>Dapper</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS T98</td>
<td>1.48</td>
<td>2.68</td>
</tr>
<tr>
<td>CS T3109</td>
<td>2.75</td>
<td>0.953</td>
</tr>
<tr>
<td>LMS T174</td>
<td>1.25</td>
<td>1.05</td>
</tr>
<tr>
<td>LMS T191</td>
<td>2.67</td>
<td>1.07</td>
</tr>
<tr>
<td>LMS T294</td>
<td>0.848</td>
<td>0.469</td>
</tr>
<tr>
<td>LMS T466</td>
<td>0.807</td>
<td>3.53</td>
</tr>
<tr>
<td>LPS T2500</td>
<td>2.05</td>
<td>3.14</td>
</tr>
<tr>
<td>OSA T1898</td>
<td>2.31</td>
<td>3.02</td>
</tr>
<tr>
<td>OSA T6357</td>
<td>2.47</td>
<td>0.943</td>
</tr>
<tr>
<td>SCS T7431</td>
<td>0.973</td>
<td>0.178</td>
</tr>
<tr>
<td>CS T2355</td>
<td>6.46</td>
<td>6.56</td>
</tr>
<tr>
<td>CS T2744</td>
<td>0.316</td>
<td>0.666</td>
</tr>
<tr>
<td>FBS T3946</td>
<td>3.76</td>
<td>0.707</td>
</tr>
<tr>
<td>FBS T4436</td>
<td>0.849</td>
<td>1.05</td>
</tr>
<tr>
<td>FBS T6390</td>
<td>1.81</td>
<td>0.447</td>
</tr>
<tr>
<td>LMS T374</td>
<td>0.580</td>
<td>1.76</td>
</tr>
<tr>
<td>LMS T3863</td>
<td>0.275</td>
<td>0.391</td>
</tr>
<tr>
<td>MFH T1913</td>
<td>0.952</td>
<td>0.341</td>
</tr>
<tr>
<td>MFH T5087</td>
<td>0.389</td>
<td>1.49</td>
</tr>
<tr>
<td>MFS T6251</td>
<td>0.966</td>
<td>1.09</td>
</tr>
<tr>
<td>RD T395</td>
<td>1.03</td>
<td>0.627</td>
</tr>
<tr>
<td>SCS T548</td>
<td>0.804</td>
<td>0.208</td>
</tr>
</tbody>
</table>
Discussion

Traditionally, sarcoma therapy has been selected on the basis of non-molecular considerations such as tumor type, grade and stage. As a result, treatments for sarcomas have not developed at the same pace as those for other more common tumors, and today we still accept with fatalism that a fraction of sarcoma patients will respond to a particular therapy, whereas others will not. We also accept that we have little control over which outcome will prevail in a given case. New approaches are desperately required to tailor treatments to the individual patient, select from current therapeutic possibilities, predict therapeutic response and develop novel targeted therapeutic modalities. Previous studies conducted in our laboratory have demonstrated a GEP in sarcoma cell lines that is unique to cell lines, which undergo apoptosis when treated with dasatinib at low nanomolar doses. In addition, we have also identified a molecular signature that successfully predicts response to dasatinib in sarcoma cell lines. In this third aim, we further investigated the clinical significance of these findings by demonstrating the expression of the molecular signature in patient tissue samples from 22 diverse sarcoma specimens.

Intriguingly, this signature theoretically predicted 10 out of 22 tumors to be responders (Table 16). The 22 gene molecular signature was not able to successfully cluster all of the tumors according to potential response, however there is promise because many of the tumors classified with their respective response groups. The fact that a signature, which was derived from cell lines, can be found in the tumors lends significant evidence for further research into the possibility of this signature to serve as a predictor of response in sarcomas.
While the focus of this dissertation has been placed on the expression of a molecular signature that predicts response to dasatinib, the reverse could also be tested. A signature that predicts non-response has equally been generated and proven in cell lines. Furthermore, it is expressed in primary sarcomas as well. The flipside to these studies could provide just as much help in designing future studies and identifying other potential therapeutic targets. Either way, the molecular signature of the responders or non-responders can be used to theoretically predict tumors that require Src activity for survival and/or metastasis.

There are several theories that could explain the discrepancies between the GEP of the cell lines and tumors and why there is not a complete correlation with potential response and GEP. One possibility to explain this discrepancy includes the immense differences between gene and protein expression that are inherent when comparing cell lines and tumors. Cell lines are grown independent of a microenvironment and lack interaction with other biological components. Tumors interact with a complex microenvironment and are exposed to a considerable amount of stimuli on an infinite level. These interactions alone (or the lack of) may account for the discrepancies between the GEP. In addition, cell lines may lack the expression of metabolic enzymes present in tumors. Tumors have been shown to become resistant to therapy with time, as in the case with gleevec and GISTs. Cell lines may lack the ability to increase the metabolism of a drug of choice because of the inability of the cells to adapt to their environment as is the case with some tumors. Moreover, it is important to account for the ability of a tumor to become resistant to a therapy as a consequence of altering the expression of genes encoding metabolizing proteins even when a signature is expressed.
The purity of the tumors specimens utilized for the microarrays are also of some concern. While, we tried to utilize samples that are as pure as possible, sarcomas by nature are heterogeneous both within the tumor sample and among patients. Sarcomas can be comprised of a variety of cellular types including stromal cells, blood vessels and other undifferentiated cells. The lack of homogeneity of cell type can dilute the signature in the tumor specimens. There have been advancements in technology which aim at improving the purity of patient specimens, such as laser capture microdissection. This would be a wonderful technique to utilize, however it just was not feasible for this study. The heterogeneity within tumors between sarcoma patients is also a possible explanation for the molecular signature discrepancies. While, there have been many consistent mutations and translocations identified in sarcoma specimens, there are still a considerable amount of diversity among sarcomas, even within the same tumor type. Plus, it is very difficult to compare tumors among different patients. The diversity among tumor GEP is as great as the diversity among people in general. However, when a molecular signature of commonality identified in cell lines can be extracted from a tumor set as diverse as ours, there is great promise and encouraging evidence to suggest that this signature may be upheld in sarcomas.
Conclusions

Three independently synthesized Src kinase inhibitors were evaluated in these studies. While the three compounds were each designed to target the catalytic domain of Src, the data generated using dasatinib have presented the most convincing preclinical data to complete further studies with this compound. Dasatinib inhibits Src mediated cellular migration and invasion in all of the Src activated cell lines examined in these studies, however only a subset of bone derived sarcoma cell lines underwent apoptosis in response to dasatinib. These observations suggest that Src associates and interacts with different molecular pathways in sarcoma cell lines. In the bone sarcoma cell lines, Src mediates an induction of a FAK-Cas-migration/invasion/survival pathways, while in the STS cell lines Src initiates FAK-Cas-migration/invasion only pathways. These findings lend evidence to suggest that Src interacts with diverse signaling molecules which varies with sarcoma type.

The dasatinib data have shown cell lines that respond to dasatinib by induction of apoptosis can be identified using a 22 gene molecular signature. Eleven of 12 human sarcoma cell lines express constitutively activated Src kinase and respond to dasatinib by inhibition of Src activation and signaling as measured by Western blot analysis and migration and invasion studies. Of these cell lines, six, which were all derived from bone sarcomas, were identified as responders to dasatinib as defined by induction of apoptosis.
Moreover, blockade of c-Src expression in this subset of sarcoma cells by silencing RNA induces apoptosis, consistent with the critical role of Src-mediated sarcoma cell survival.

Microarray analysis and hierarchical clustering allowed us to identify a 26 probe set list that successfully predicts response to dasatinib. To test the reliability of the molecular signature two cells lines with unknown response to dasatinib were used. Independent analysis of both the GEP and the molecular response to dasatinib of the cells were compared. The molecular signature that predicts response successfully characterized the cell lines into their respective groups, one cell line, HOS, was a responder and the other, SW1353 was a non-responder. Here, we show a molecular signature predicting response to dasatinib by induction of apoptosis in cell lines.

Furthermore, we performed microarray analysis on 22 human sarcomas of varying sarcoma histology to determine whether components of the molecular signature, Ephrin-A1 or Dapper expression could be used to characterize the tumors based on potential response to dasatinib. A list of possible responsive and non-responsive tumors was generated based on their GEP. When clustered based on the expression of the 26 probe set molecular signature or Ephrin-A1 and Dapper, the clustered provided encouraging results to suggest that the signature has promise to predict response in tumors. While not all of the sarcomas clustered with their respective groups (potentially, responsive or non-responsive), many of the tumors did cluster with their groups and the predictive trends of Ephrin-A1, Dapper and the molecular signature are up-held. These data suggest that our signature could become useful for the identification of patients eligible for new therapeutic options.
Three of the four genes from the molecular signature further validated by qRT-PCR analysis were upheld as reliable predictors of response; Histone H3, FAF1 and δ-catenin. However, Ephrin-A1 and Dapper were less reliable as predictive markers as reversed expression pattern was evident in these cell lines. The six genes that were validated by qRT-PCR; Histone H3, FAF1, α-catenin, δ-catenin, Ephrin-A1 and Dapper, are all thought to play unique roles in cancer. The functions these genes play in the malignant phenotype lend evidence to further investigate the role of these genes in sarcomas.

Histone H3 and FAF1 were upregulated in the cell lines that respond to dasatinib. Many types of cancer are associated with translocations or mutations in chromatin-modifying enzymes and regulatory proteins. Histones are integral components of the machinery responsible for regulating gene transcription. Phosphorylation of Histone H3 plays important regulatory signaling roles in the processes of chromosome condensation during mitosis and transcription following external stimulation of gene expression growth factors or stress. Histone H3 is phosphorylated in serine 10 and 28. Fyn, one of many kinases that phosphorylate Ser10. The phosphorylation of Ser10 on Histone H3 is critical for EGF-induced neoplastic cell transformation. This evidence suggests that Histone H3 may serve as a critical target for cancer therapy (278). Interestingly, the overexpression of Histone H3 was upheld in many of the tumors categorized as potential responders. Histone H3 overexpression in the responsive group may not be easily explained in the context of these studies. However these studies have identified Histone H3 as an overexpressed gene which provides evidence to further investigate its activity in sarcomas and possibly evaluated it as a therapeutic target.
While FAF1 overexpression was only upheld in the cell lines, this may be caused by the inherent difference in phenotype between cell lines and tumors. FAF1 is a pro-apoptotic factor that is involved with Fas-mediated caspase 8 cleavage and induction of apoptosis (279). The FAF1 protein expression has been demonstrated to be reduced in gastric carcinomas (280). One possible explanation for the overexpression of FAF1 in responsive cell lines may cause the cells to be more sensitive to induction of apoptosis as compared to the non-responsive cell lines. Furthermore, the function of FAF1 has been evaluated only in the context of cell lines, the role of FAF1 may not be consistent in tumors.

Two catenins were underexpressed in the responsive cell lines; α- and δ-catenin. The expression of these two catenins were less clear cut in the tumors, however there is evidence to suggest that they may play important function in tumorigenesis. The formation of tissues and organs largely depends on interactions between neighboring cells. These associations orchestrate the assembly of diverse cell types into organized patterns to establish a complex organism. These interactions also permit adult tissues to perform unique functions, preserve architectural integrity and precisely coordinate the events that enable cells to remodel tissues during normal homeostasis and to synchronize in response to injury to repair tissues. By communicating signals through adhesion receptors, cells can respond and elicit the spatially coordinated events needed to maintain tissue homeostasis. The imbalance of which is frequently observed in the malignant phenotype. Catenins are an intracellular anchor proteins that attach cadherins to actin fibers within the cell and aid in the formation of adherens junctions (AJ), the building blocks of cellular architecture. α- and δ-catenin are two important AJ catenins that
integrate cell to cell junctions and regulate cytoskeletal dynamics by governing signaling pathways involved in morphogenesis, homeostasis and intercellular communication between different cell types within a given tissue (281).

α-catenin binds indirectly to cadherins via interactions with β-catenin, a protein commonly mutated in cancers. Recently, α-catenin has also been found to be an essential in coordinating actin dynamics and inversely correlating cell adhesion with proliferation (282). It is hypothesized that loss of α-catenin may account for the absence of calcium-dependent cell-cell adhesion in cancers with intact E-cadherin expression. In addition, CTNNA1, the gene that encodes α-catenin, has been shown to be a tumor suppressing gene (283-285). Furthermore, inactivating mutations of CTNNA1 have been demonstrated in lung, prostate, ovarian, and colon cancer cell lines (286). These cell lines lack normal cell-cell adhesion because the cadherin-catenin interactions are disrupted. IHC has also demonstrated a loss of α-catenin expression in primary tumors (286). Mutational analysis has also shown that α-catenin expression is decreased in synovial sarcomas (287). Therefore, α-catenin is considered as an invasion suppressor molecule in cancers. The expression of α-catenin was relatively low in all of the sarcoma specimens. This finding may provide evidence to further explore the role of α-catenin in sarcomas. Thus far, α-catenin mutations have been identified in synovial sarcomas, perhaps the expression of α-catenin is so low in the microarray analysis because more sarcoma possess mutations in this gene.

While the responsive cell lines had low levels of δ-catenin expression, the responsive tumors had higher levels as compared to the non-responsive tumors. These findings could lend evidence to suggest differential roles for δ-catenin in cell lines and
tumors and could be due to the lack of microenvironment in the cell lines. A role for δ-catenin in cancer has been demonstrated in the literature. Unlike α-catenin whose sequence is considerably different from other catenins, δ-catenin is closely resembles β-catenin. Originally identified as a Src substrate, δ-catenin associates with cadherin-mediated AJs, thus stabilizing them at the membrane. IHC studies have reported loss of delta-catenin in some primary tumors, however mutation of the CTNND1 gene is extremely rare (286).

Ephrin-A1 and Dapper were two other genes identified as potential biomarkers that predict response. Both genes were upregulated in the responsive cell lines. However, the predictability of response governed by Ephrin-A1 and Dapper expression was not successful when they were tested using the HOS and SW1353 cell lines. On the other hand, Ephrin-A1 and Dapper were overexpressed in the responsive group of tumors compared to the non-responsive group. Furthermore, a role of Ephrin-A and Dapper in cancer has been described.

The Eph family of receptor tyrosine kinases and their cell-presented ligands, the ephrins, are overexpressed in a variety of cancers, including breast and gastrointestinal cancers, melanomas and neuroblastomas. Upon ephrin binding, the Eph receptors are phosphorylated at specific tyrosine residues in the cytoplasmic region, which then serve as docking sites for various signaling molecules. Many SH2 domain-containing proteins have been found to interact with the phosphotyrosines of activated Eph receptors, including Src and Fyn. These interactions have been implicated in cell adhesion, cell motility, and cytoskeleton rearrangement. However, the mechanisms by which
individual molecules and signaling pathways exert specific functions has yet to be described (288).

Ephrin-A1 is a ligand of EphA2 and is involved in the initiation of cellular migration, functions as an angiogenic factor \textit{in vitro} and is essential for TNF-\(\alpha\)-induced angiogenesis. Its expression in tumors correlates with initiating invasion by attracting endothelial cells. In mice models using Karposi-sarcoma-derived tumors as xenografts, the vasculature invading the tumors expressed increased ephrin-A1 and EphA2 (289). Further, ephrin-A1 and EphA2 can be detected on the surface of endothelial cells in a variety of human tumors.

Dapper1, gene mapped to human chromosome 14q22.3 and is deleted in astrocytoma, was identified as an interacting protein for Disheveled, a Wnt signaling mediator, and modulates Wnt signaling. WNT signals play key roles in carcinogenesis and embryogenesis through the specification of cell fate and polarity. Dapper has been described as an antagonist Wnt signaling by inducing Disheveled degradation (290). Consequently, based on the importance of the WNT signaling pathway in cancer, Dapper is predicted to be potent cancer-associated gene (291).

While Ephrin-A1 and Dapper were not successful at predicting the response of HOS and SW1353 to dasatinib, the expression of these genes in sarcomas may be of interest for further studies. Both have significant roles in cancer, and may have a vital function in sarcomas. Furthermore, EphA1, the receptor for Ephrin-A1, is a known target of dasatinib. Perhaps the cell lines have provided a vital clue into a significant biomarker of predicting dasatinib prediction not only in cell lines, but in tumors as well. Only future studies can tell.
One must keep in mind that this signature that predicts response to dasatinib by induction of apoptosis in sarcoma cell lines is completely based on a genomic expression analysis. The information gained from a genomic analysis using microarrays is highly informative, however does not portray the functional events occurring within the system analyzed. A GEP provides a snapshot of the genes which are expressed at a given point of time. In comparison, a proteomic analysis would provide a more informative understanding of the actual events occurring within a system as dictated by the gene products. Identifying a protein expression profile would present a functional analysis of the genes expressed and more directly translate the relevant overall effects of the biology at play within a system and more precisely predict cellular behavior. The information lost in the translation between gene expression and protein expression may account for many of the inconsistencies observed throughout the signature analysis within the cell lines and tumors analyzed in these studies. Nevertheless, the identification of a molecular signature that has been proven to predict response to dasatinib in cell lines is an excellent starting point for generating more sophisticated analyses aimed at further identifying a more precise and accurate signature that effectively predicts response to dasatinib in human sarcoma patients.

In conclusion, Src activity is essential for the survival of a subset of sarcomas. We have identified a molecular signature that can predict the response of cell lines to dasatinib. The molecular signature can successfully identify cell lines that will undergo apoptosis upon inhibition of Src activity by dasatinib. This molecular signature is also present to some degree in primary human sarcomas and should be further validated to determine the efficiency of this signature to predict patient response to dasatinib.
Together, these studies reveal that both constitutive Src activation and the expression of a molecular signature that predicts response to dasatinib in cell lines may potentially play a critical role in the response of sarcomas to dasatinib. Thus, novel therapeutic approaches that inhibit Src signaling in sarcoma that express the molecular signature may have the potential to induce apoptosis and sensitize tumors to chemotherapy.
Clinical Significance

TKIs have become a viable therapeutic option for the treatment of sarcomas eliciting aberrantly activated tyrosine kinase signaling cascades. Cell line based *in vitro* and *in vivo* models have demonstrated mixed, yet promising potential uses of TKIs as single agent or in combination with current treatment options. Proof-of-principle of this concept has been obtained for sarcomas in the case of Gleevec treatment of GISTs and DFSPs.

Our data offers preclinical evidence to further investigate the response of sarcomas to dasatinib in clinical trials. In that, dasatinib is a promising therapeutic option for treating sarcomas with activated Src kinase because it may prevent metastasis by inhibiting tumor cell migration and invasion, as well as induce apoptosis in a subset of bone derived sarcomas. A molecular signature in the bone subset of sarcomas that predicts apoptosis response to dasatinib has been identified in these studies. The predictive molecular signature of apoptosis response to dasatinib represents a testable hypothesis in clinical trials.

TKIs currently in early phase trials have provided promising therapeutic options for treating sarcomas. Further work is required to delineate the role TKs play as effectors of tumorigenesis in sarcoma and differentiate between relevant and irrelevant mutations with regards to upstream and downstream signaling. To be effective, a TKI must target a
pathway which is essential and vital for the survival, as in the case of c-KIT in GIST and PDGFR in DFSP and Src in OSA cell lines.
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