Inhibition of Src Phosphorylation Alters Metastatic Potential of Osteosarcoma In vitro but not In vivo

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Abstract Purpose: Pulmonary metastasis remains the major cause of mortality in osteosarcoma. Src tyrosine kinase is a key player involved in metastatic pathways in multiple human cancers. c-Src has been shown to be expressed and phosphorylated in osteosarcoma cell lines and inhibiting Src phosphorylation in these cells causes inhibition of the metastatic phenotype In vitro. We studied the effect of inhibition of Src phosphorylation in preventing the growth and development of pulmonary metastases in osteosarcoma.

Experimental Design: Dasatinib, a dual Src-Abl kinase inhibitor, was used to study the effect of Src kinase inhibition on proliferation, adhesion, and invasion of osteosarcoma cell lines In vitro and in preventing the development of pulmonary metastases in a spontaneous metastatic mouse model.

Results: In vitro, phosphorylation of Src and its downstream signaling molecules such as focal adhesion kinase, Crk-associated substrate, and c-Jun was inhibited at nanomolar concentrations of dasatinib. Dasatinib was not cytotoxic against the osteosarcoma cells with the IC50 ranging from 10 to 20 μmol/L but effectively inhibited the adhesion and migration of osteosarcoma cells at 10 to 100 nmol/L. However, in vivo, dasatinib did not inhibit the development of pulmonary metastases despite complete inhibition of Src phosphorylation in the primary tumors. No effect was seen in the primary tumor growth and the degree of apoptosis.

Conclusions: These results suggest that Src kinase activation might not be the primary pathway involved in the development of pulmonary metastases in osteosarcoma.

Osteosarcoma is the most common primary malignant bone tumor in children and young adults. There are ~600 new cases of osteosarcoma in the United States each year. Despite improvements in local control, mortality rates in osteosarcoma have changed little in the past 30 years. The overall survival rate remains ~60% to 70%. Survival rates in patients with pulmonary metastases are ~20% to 30%. Additionally, ~30% of patients with only local disease at diagnosis will relapse with pulmonary metastases (1). Disease metastatic to the lung remains the single most significant poor prognostic feature in osteosarcoma. Novel therapies are needed to decrease the incidence and development of pulmonary metastases and to increase cure rates. Targeted therapies in conjunction with conventional chemotherapy agents might help achieve these goals.

Src is a nonreceptor tyrosine kinase encoded by the c-Src proto-oncogene. It is one of the nine members of the Src family of kinases. Both overexpression and overactivation of c-Src has been shown to aid in the development of cancer. Src kinase activity is regulated via a variety of receptor tyrosine kinases such as epidermal growth factor receptor tyrosine kinase, platelet-derived growth factor tyrosine kinase, and integrin receptors (2–4). It is also regulated by phosphorylation at the negative COOH-terminal Tyr527 residue, which renders it inactive (5). Activation of Src kinase leads in turn to activation of a variety of downstream signaling pathways such as the Ras/mitogen-activated protein kinase pathway, and activation of the transcription factor STAT-3, resulting in cell cycle progression from G2 to M phase and vascular endothelial growth factor production, which aids in angiogenesis and tumor growth and invasion (6–8). Further, Src regulates the stability of focal adhesions through its interaction with focal adhesion kinase (FAK). c-Src expression leads to disruption of focal adhesions by causing disruption of the catenin-cadherin complexes, thus contributing to the invasive capability of the tumor cells. FAK signaling mediated by c-Src leads to activation of c-Jun kinase, which further promotes invasion via regulating matrix metalloproteinases (9). Thus, c-Src activation plays an important role in cancer cell survival, growth, adhesion, and invasion and leads to an overall aggressive and metastatic phenotype.
Dasatinib is a novel dual Src-Abl kinase inhibitor that is Food and Drug Administration approved for imatinib-resistant chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia in adults (10). Its activity in chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia is primarily due to the inhibition of Bcr-Abl tyrosine kinase. It is an orally active compound with a favorable toxicity profile (11). It has its activity in chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia in adults (10). Its activity in chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia is primarily due to the inhibition of Bcr-Abl tyrosine kinase. It is an orally active compound with a favorable toxicity profile (11). It has also been shown to inhibit various c-Src-mediated downstream pathways in a variety of malignant cell lines such as prostate, head and neck, and lung cancer cells and in a variety of sarcoma cells, thus affecting their proliferation, adhesion, and migration capabilities (12–15). These in vitro data indicate that it might be a promising therapeutic agent in preventing growth and metastasis of a variety of human cancers. We show that Src is a promising therapeutic agent in preventing growth and metastasis in our in vivo model of osteosarcoma. In addition, Src is minimally phosphorylated in the lung metastases between the control and the treated groups. These data suggest that Src kinase pathway might not be the primary pathway involved in the development of pulmonary metastases in this model of osteosarcoma. Hence, the development and use of targeted therapies to inhibit Src kinase might not be a useful strategy to prevent pulmonary metastases in clinical settings as might have been suggested by the in vitro data alone.

Materials and Methods

Cells and reagents. SaOS-2, HOS, and 143B cell lines were obtained from the American Type Culture Collection. Cells were grown and maintained in modified Eagle’s medium containing 10% fetal bovine serum, 0.5% penicillin-streptomycin, and 1% glutamine at 37°C with 5% CO₂. Dasatinib was provided by Bristol-Meyers Squibb Pharmaceutical Research Institute. Stock solution of 10 mM dasatinib was prepared in DMSO and stored at −20°C.

Antibodies to Src, phospho-Src (Y419), FAK, phospho-FAK (Y576/ Y577), β-actin, and phospho-p130CAS were obtained from Cell Signaling Technology. Antibody to phospho-p130CAS was obtained from Santa Cruz Biotechnology.

Cytotoxicity assay. SaOS-2 and 143B cells were plated in 96-well flat-bottomed microtiter plates at a concentration of 500 per well in 100 μL medium. After 24 h of incubation at 37°C, cells were treated with increasing concentrations of dasatinib and incubated for 72 h. After 72 h, 250 μg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide obtained from Sigma was added to each well and incubated at 37°C for 6 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was converted to formazone crystals by mitochondria of the viable cells, which were then dissolved in 100 μL DMSO. Absorbance was measured at 595 nm using a Versamax spectrophotometer (Molecular Devices). Viability was calculated as the absorbance of drug-treated cells divided by the absorbance of vehicle-treated cells. The IC₅₀ was defined as the drug concentration at which absorbance was 50% of that of the controls.

Cell adhesion assay. The 143B, HOS, and SaOS-2 osteosarcoma cell lines were treated with dasatinib for 24 h. Cells were then plated in fibronectin-coated 96-well plates (BD Biosciences) for 1 h. Nonadherent cells were removed by washing with serum-free medium thrice. Adhered cells were fixed with methanol for 10 min and stained with crystal violet. Plates were analyzed with the Versamax spectrophotometer at 540 nm.

Cell migration assay. Cells were serum starved for 18 to 24 h before the assay. 24-well plates with polycarbonate membrane inserts (Chemicon International) were used for the assay. Briefly, cells were harvested and suspended in serum-free medium at the concentration of 1 × 10⁶/mL. Cells were then incubated with increasing concentrations of dasatinib for 30 min at 37°C. The cell suspension (300 μL) was added to the inner chamber of the insert; serum-rich medium (500 μL) was added to the outer chamber and the plates were incubated at 37°C with 5% CO₂ for 24 h. After 24 h, the inner chambers were removed and placed in the cell staining solution for 20 min. Excess stain was washed off by dipping the inserts in water. The inner surface of the inserts was swabbed with cotton-tipped applicator to remove the cells that did not migrate. The outer surface of the insert, containing the migrated cells, was dipped in 400 μL extraction buffer for 10 min. Absorbance of the solution, as a measure of degree of migration of cells, was measured at 540 nm using the Versamax spectrophotometer.

Western blotting. Protein was collected from 143B cells treated with increasing concentration of dasatinib (0–3,000 nmol/L) for 24 h. 20 μg protein from each sample was resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in Tris buffered saline containing 0.1% Tween 20. Antibodies to SRC, phospho-SRC, FAK, phospho-FAK, p130CAS, phospho-p130CAS, C-Jun, and phospho-C-Jun antibodies. Blots were analyzed using Amersham ECL Plus Western blotting detection reagents.
TBS-Tween 20 and then incubated with various primary antibodies overnight at 4°C. Horseradish peroxidase-conjugated anti-rabbit secondary antibody (Cell Signaling Technology) was used to detect protein-bound primary antibody. Bound secondary antibody was detected by using Amersham ECL Plus Western blotting detection reagents (GE Healthcare).

Green fluorescent protein tagging of cells. 143B cells were transfected with pAcGFP-C1 (Clontech) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, the top 5% of green fluorescent protein (GFP)-expressing cells were sorted by fluorescence-activated cell sorting. Sorted cells were plated in 24-well plates and selected in G418 (0.75 mg/mL). The selected clones were expanded and pooled.

Animal experiments. Five- to 6-week-old female C.B-17 severe combined immunodeficient mice were used for the in vivo experiments and the spontaneously metastatic mouse model was developed as described previously (16). Briefly, 143B cells were harvested and suspended in PBS at a concentration of 1 × 10⁷/mL. Mice were anesthetized using isoflurane and 0.1 mL of the cell suspension was injected into the right tibia of each mouse. Tumors were allowed to grow for the next 4 to 6 weeks. Two to three mice were sacrificed weekly starting at 2 weeks after injection and the lungs were harvested and fixed in formaldehyde. Serial lung sections were cut and stained with H&E to identify metastases by light microscopy.

After the first experiment confirming the development of spontaneous pulmonary metastases, mice were divided into control and treated groups (8-10 mice per group) and the following sets of experiments were conducted to evaluate the activity of dasatinib in preventing the development of pulmonary metastases: (a) dasatinib oral 50 mg/kg dose twice a day for 4 weeks (17) starting 7 days after injecting the tumor cells in the tibia, (b) dasatinib oral 50 mg/kg/dose twice a day for 4 weeks starting 2 days before injecting the tumor cells, and (c) dasatinib intraperitoneal 25 mg/kg/dose once a day for 4 weeks starting 2 days before injecting the tumor cells. The control groups received the vehicle for the duration of each experiment. Serial primary tumor volumes were measured during the course of the experiment using the formula: \[ \pi \times \left( \frac{d}{2} \right)^2 \], where \( d \) is the diameter of the tumor. Mice were sacrificed at the end of treatment. Primary tumors and lungs were harvested from each mouse in both groups for histopathology and immunohistochemistry.

Similar experiments were repeated with GFP-tagged 143B cells whereby the tissues were fixed with 4% paraformaldehyde and analyzed under fluorescent microscopy. In these mice, left lungs were used for fluorescent microscopy and right lungs were used for histopathologic analyses. Lungs were grossly assessed under dissecting microscope for GFP-positive metastases and subsequently sectioned and analyzed for GFP-positive micrometastases.

Histopathology and immunohistochemistry. Primary leg tumors and right lungs of all mice from both the control and the treated groups were harvested at the end of the treatment and fixed with formaldehyde. The fixed tissue was sectioned and histologic analyses were done using H&E staining. Serial lung sections were analyzed under light microscopy for presence of metastases under low-power fields and then confirmed under high-power fields. In addition, both the primary tumor sections and the lung metastases from the control and treated mice were analyzed for total Src and phospho-Src expression using the Src and phospho-Src (Y419) antibodies, respectively, according to the manufacturer's protocol.

Fig. 2. In vitro assays: dasatinib does not inhibit the growth of 143B and SaOS-2 cells in vivo but effectively inhibits the adhesion and migration capability of 143B, HOS, and SaOS-2 cells in vivo at nanomolar concentrations. A, cytotoxicity assay: cells were treated with increasing concentration of dasatinib in 96-well plates for 72 h. Viability was then assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and represented as absorbance of treated cells/absorbance of control cells. B, cell adhesion assay: dasatinib cells were treated with increasing concentrations of dasatinib for 24 h and evaluated for their adhesion capability using the fibronectin coated 96-well plates. C, cell migration assay: serum-starved 143B, HOS, and SaOS-2 cells were treated with increasing concentrations of dasatinib for 30 min and evaluated for their migration capability using polycarbonate membrane inserts. Results for both assays were represented as the absorbance measured at 540 nm using a Versamax spectrophotometer.
Apoptosis assay in vivo. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay was done on paraffin-embedded tissue sections using the apoptosis detection kit (Chemicon International). Primary leg tumors from the control and the treated mice were harvested at the end of treatment and fixed with formaldehyde. Paraffin-embedded tissue sections were deparaffinized with xylene and serial washes of ethanol. Endogenous peroxidase was quenched with 3% hydrogen peroxide. The slides were incubated with terminal deoxynucleotidyl transferase enzyme for 1 h at 37°C. The reaction was stopped with the stop buffer provided in the kit. Anti-digoxigenin conjugate was added for 30 min at room temperature. Slides were then washed with PBS and peroxidase substrate was added for 5 min at room temperature. Slides were counterstained with hematoxylin and then mounted with a coverslip. Percentage of apoptotic cells was determined by counting the number of brown-stained nuclei per high-power field of a light microscope. Results represent an average of six high-power fields.

Statistical analyses. In vitro, the effect of increasing concentration of dasatinib on adhesion and migration of cells was compared by the $t$ test. In vivo, the primary tumor volume was compared between the control and the treated groups for each experiment by using the $t$ test. The number of mice carrying lungs metastases was manually counted in serial histopathologic sections of lungs and the average was calculated per group.

Results

Dasatinib inhibits Src-mediated signaling pathways in vitro. Dasatinib inhibits Src kinase-mediated downstream signaling pathways in vitro (Fig. 1). 143B cells treated with increasing concentrations of dasatinib were analyzed for phosphorylation of Src and its downstream targets such as FAK, Crk-associated substrate (p130CAS), and c-Jun. Phosphorylation of Src at Tyr416 was completely inhibited by dasatinib. Further, phosphorylation of FAK at Tyr576/Tyr577 and p130CAS was also completely inhibited by dasatinib. Phosphorylation of c-Jun was inhibited at higher concentrations of dasatinib ranging from 1,000 to 3,000 nmol/L. These results suggest that dasatinib inhibits activity of Src kinase and its major downstream signaling pathways.

Fig. 3. Primary tumor growth: dasatinib does not inhibit tumor growth in vivo. Serial tumor volumes were measured at the primary site for the duration of each experiment in the control and the treated groups and compared using the $t$ test. Comparison of mean tumor volumes between the two groups.

Fig. 4. Histopathologic and fluorescent microscopy analyses of pulmonary metastases: dasatinib did not inhibit the development of pulmonary metastases in mice with 143B xenografts irrespective of when therapy was started. A, representative H&E section showing pulmonary metastasis under light microscopy (magnification, ×400). B, representative image of lung under a dissection microscope showing GFP-positive metastasis. C, representative image of a section of lung metastasis under a confocal microscope. Multiple sections of right lung from each control and treated mouse were stained with H&E and analyzed under low-power field (magnification, ×10) for metastases that were then confirmed under high-power field (magnification, ×400). Similarly, left lung from each control and treated mouse was analyzed grossly under a dissection microscope and multiple sections were analyzed under fluorescent light and confocal microscope for GFP-positive tumor cells.
Dasatinib inhibits cell adhesion and migration in vitro but not cell proliferation. Dasatinib was not effective in inhibiting the growth of osteosarcoma cell lines in vitro. The IC_{50} of dasatinib was between 10 and 20 μmol/L for both SaOS-2 and 143B lines (Fig. 2A). This concentration is significantly higher than the physiologically achievable plasma trough concentration of 100 nmol/L at the current dosing schedules (18). However, at nanomolar concentrations, dasatinib inhibited adhesion of the osteosarcoma cells to a fibronectin matrix in a dose-dependent manner at nanomolar concentrations. In all three osteosarcoma cell lines, the number of cells adhered to the fibronectin matrix was significantly decreased after 24 h of treatment with dasatinib at concentrations of 10 to 100 nmol/L (Fig. 2B). Tumor cell migration was also inhibited in a dose-dependent manner by dasatinib. After 24 h of treatment, the migration of cells was significantly decreased at concentrations of 10 to 100 nmol/L (Fig. 2C). Inhibition of Src phosphorylation by dasatinib results in expected alterations in in vitro cell adhesion and migration but is not sufficient to inhibit cell growth at physiologically relevant concentrations.

Dasatinib inhibits neither the growth of the primary tumor nor the development of pulmonary metastases in vivo. No significant difference was noted in the primary tumor growth between the control and the treated groups. The mean tumor volume was 104 ± 44 mm³ in the control group and 81 ± 28 mm³ in the treated group (P = 0.09; Fig. 3). There was no difference in the number of mice that developed pulmonary metastases between the control and the treated groups in all the three sets of experiments with five to six mice per group developing metastases in each experiment (Fig. 4). These data were comparable in both GFP-transfected and nontransfected 143B cell primary tumors. We initially speculated that Src may be involved in tumor spread but not the growth of tumors in situ. However, dasatinib was also ineffective at preventing pulmonary metastases despite starting the treatment before the tumor implantation.

Dasatinib does not increase apoptosis in vivo. Dasatinib did not increase the degree of apoptosis in the treated primary tumors compared with the controls (Fig. 5A and B). The mean number of brown-stained nuclei per high-power field was 17 ± 3 in the control group and 15 ± 4 in the treated group (Fig. 5C).

Dasatinib inhibits phosphorylation of Src in vivo. Oral administration of dasatinib to tumor-bearing mice completely inhibited phosphorylation of Src in the primary tumor without a change in the total Src expression (Fig. 6A-D). In the lung metastases, interestingly, the expression of phospho-Src was significantly decreased in both the control and the treated groups with no difference in expression between the two groups (Fig. 6E-H). Taken together, these data show that despite complete inhibition of Src phosphorylation in the primary tumor by dasatinib, metastatic spread of the 143B tumor cells is not inhibited. In fact, constitutive Src phosphorylation does not appear to contribute to growth of spontaneous pulmonary metastases.

Discussion

In the current study, we hypothesized that dasatinib, a potent inhibitor of Src kinase, would be an effective antimetastatic agent in osteosarcoma. We show that dasatinib effectively inhibited adhesion and migration of osteosarcoma cell lines in vitro at physiologically achievable concentrations (10-100 nmol/L). Dasatinib inhibited phosphorylation of Src as well as downstream signaling molecules. These results are consistent with previously published data in other sarcoma cell lines (15).
However, dasatinib did not inhibit the development of pulmonary metastases in vivo using a orthotopic model of spontaneous pulmonary metastases with the 143B cell line despite inhibition of Src phosphorylation in the primary tumor. These data in conjunction with the minimal constitutive expression of phospho-Src in pulmonary metastases suggest that Src kinase activity may not be an essential pathway responsible for development of metastases following orthotopic injection of the 143B cell line. In addition, dasatinib had no significant effect on cell proliferation and viability in vitro. The IC_{50} of 10 to 20 μmol/L (1,000 times that needed to inhibit Src phosphorylation) suggests cytotoxicity possibly due to potential off-target effects instead of the direct inhibition of Src kinase activity. In vivo, no significant difference in the degree of apoptosis in 143B xenografts was noted between the control and the treated mice despite clear inhibition of Src phosphorylation.

Shor et al. (15) reported that dasatinib is able to diminish the metastatic phenotype of sarcoma cell lines in vivo. We confirm these in vivo results in three osteosarcoma cell lines. Among the HOS, SaOS-2, and 143B osteosarcoma cell lines, only the 143B line metastasizes spontaneously in vivo after orthotopic injection. The in vivo effects of dasatinib in this line are encouraging and predict that Src inhibition may be of therapeutic benefit in osteosarcoma. However, the in vivo model system is not consistent with the in vitro behavior. Dasatinib had no effect on tumor growth or the development of spontaneous pulmonary metastases in vivo. Our data are limited by the fact that only one of the cell lines used for the in vivo studies metastasizes spontaneously with orthotopic injection into the tibia of a severe combined immunodeficient mouse. However, at the very least, the data suggest that the in vivo model systems used in Fig. 2 of this report, and commonly by other investigators, may not be sufficient to define the effect of a targeted chemotherapeutic agent on metastases. One may hypothesize that growth factors contained at relatively high concentrations in FCS may account for differential utilization of signaling pathways in vivo and in vitro.

Src kinase activity has been implicated in promoting key oncogenic mechanisms such as cell proliferation, adhesion, invasion, and resistance to apoptosis, thus defining it as a prometastatic pathway (9, 19–21). Inhibition of Src and its downstream signaling pathways has been shown to inhibit growth, migration, and invasion of a variety of human cancer cells. Dasatinib inhibits migration and invasion in in vitro models of sarcomas, pancreatic cancer, lung cancer, and head and neck cancer (12–15, 22). SKI-606, another dual Src-Abl kinase inhibitor, has been shown to block breast cancer invasion and migration both in vitro and in vivo (23).

The establishment of pulmonary metastases involves migration of tumor cells across the endothelial membrane in the primary tumor, tumor spread and survival in the systemic vasculature, homing and survival of the tumors in pulmonary capillaries, migration across the endothelium of pulmonary vessels, and growth of the tumor in the pulmonary parenchyma. It is possible for Src to play a differential effect on any one of these processes. Therefore, we initiated dasatinib therapy 7 days after injection of tumor cell at the primary site in the tibia as well as 2 days before. Regardless, when therapy was initiated, dasatinib had no effect on the development of pulmonary metastases, although it inhibited Src phosphorylation in the primary tumors (Fig. 6A–D). The fact that metastases formed even when dasatinib was started before the development of the primary tumor suggests that Src phosphorylation may not be required for any of the steps of the metastatic process in the 143B cell line in vivo.

Other signaling pathways, such as ezrin, CXCR4, and insulin-like growth factor-I receptor, might be the important mediators in this phenomenon. Ezrin, a membrane-cytoskeleton linker, has been shown to be necessary for osteosarcoma metastases.
via activation of mitogen-activated protein kinase and AKT/mammalian target of rapamycin pathways (24–26). High expression of ezrin in patient samples of osteosarcoma has been related with poor outcome (26, 27). Similarly, high expression of CXCR4, a chemokine, in patient samples of osteosarcoma has been linked to poor overall and metastasis-free survival. Inhibition of this pathway leads to decreased pulmonary metastases in osteosarcoma (28, 29). Recently, insulin-like growth factor-I receptor pathway via its activation of the mammalian target of rapamycin pathway has been implicated in the growth factor-I receptor pathway via its activation of the mam-

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