Targeted inhibition of Src kinase with dasatinib blocks thyroid cancer growth and metastasis

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Abstract

Purpose: There are no effective therapies for patients with poorly differentiated thyroid cancer (PTC) or anaplastic thyroid cancer (ATC), and metastasis to the bone represents a significantly worse prognosis. Src Family Kinases (SFKs) are overexpressed and activated in numerous tumor types and have emerged as a promising therapeutic target, especially in relation to metastasis. We recently showed that Src is overexpressed and activated in thyroid cancer. We therefore tested whether inhibition of Src with dasatinib (BMS-354825) blocks thyroid cancer growth and metastasis.

Experimental Design: The effects of dasatinib on thyroid cancer growth, signaling, cell cycle, and apoptosis was evaluated in vitro. The therapeutic efficacy of dasatinib was further tested in vivo using an orthotopic and a novel experimental metastasis model. Expression and activation of SFKs in thyroid cancer cells was characterized, and selectivity of dasatinib was determined using a Src-gatekeeper mutant.

Results: Dasatinib treatment inhibited Src signaling, decreased growth, and induced cell cycle arrest and apoptosis in a subset of thyroid cancer cells. Immunoblotting showed that c-Src and Lyn are expressed in thyroid cancer cells, and that c-Src is the predominant SFK activated. Treatment with dasatinib blocked PTC tumor growth in an orthotopic model by > 90% (p=0.0014). Adjuvant and post-treatment approaches with dasatinib significantly inhibited metastasis (p=0.016 and p=0.004, respectively).

Conclusion: These data provide the first evidence that Src is a central mediator of thyroid cancer growth and metastasis, indicating that Src inhibitors may have a higher therapeutic efficacy in thyroid cancer, as both anti-tumor and anti-metastatic agents.
Translational Relevance

New therapies with the potential to inhibit both tumor growth and metastasis are needed for patients with advanced thyroid cancer. The Src kinase pathway has emerged as a major player in cancer progression, especially relating to metastasis. We recently showed that the Src target, FAK, is overexpressed and phosphorylated in patient thyroid tumor samples, providing justification to pursue this pathway as a clinically relevant target. Here, we show that inhibition of Src with dasatinib blocks thyroid cancer growth and transformation \textit{in vitro}. We further show that dasatinib has high therapeutic efficacy as an anti-tumor and anti-metastatic agent \textit{in vivo}, blocking tumor growth in an orthotopic model, and metastatic progression in an experimental metastasis model. Thus, Src plays a critical role in promoting both tumor growth and outgrowth of metastases, indicating that Src inhibitors may be of particular relevance in thyroid tumors with metastatic potential, as dual anti-tumor and anti-metastatic agents.
Introduction

There are currently no effective therapies for patients with advanced thyroid cancer, which includes patients diagnosed with advanced papillary thyroid cancer (PTC) or anaplastic thyroid cancer (ATC) (1). Notably, ATC is one of the most aggressive human cancers with greater than 95% mortality at 6 months. Extrathyroidal invasion and metastasis are the most common causes of thyroid cancer-related death, and metastasis to the bone predicts a significantly worse prognosis (2). Although much effort has been devoted to decipher the mechanisms involved in the progression of this cancer, little progress has been made in the development of new therapies (1, 3).

Src family kinases (herein referred to as SFKs or Src) are a multifunctional nonreceptor tyrosine kinase family that regulates a variety of cellular processes, including growth, survival, migration, and invasion (4). SFKs regulate these pro-tumorigenic functions via activation of downstream signaling pathways, including mitogen-activated protein kinase (MAPK/ERK), phosphatidylinositol-OH kinase (PI3K), signal transducer and activator of transcription-3 (Stat3), p130Cas, paxillin, and focal adhesion kinase (FAK). SFKs are overexpressed and/or activated in many tumor types (5-10). Of the nine SFK members, c-Src, Fyn, and Yes are most widely expressed, and c-Src itself has been most frequently implicated in tumorigenesis and metastasis (11). c-Src has been shown to play an important role in regulating osteoclast function and tumor colonization to the bone, making Src an attractive therapeutic target for the prevention and treatment of bone metastases (12, 13). While the remaining SFK members are expressed primarily in cells of hematopoietic origin, recent studies have shown that Lyn, Fyn, and Fgr are expressed and activated in epithelial-derived cancers (14-17).

Because SFKs plays a central role in the regulation of numerous pro-tumorigenic pathways, the development of pharmacologic inhibitors targeting the Src pathway is an active
area of investigation. Clinical trials are underway testingSrc inhibitors in solid tumors, including BMS-354,825 (dasatinib; Bristol-Myers Squibb), bosutinib (SKI-606, Wyeth) (18), and AZD0530 (saracatinib; AstraZeneca) (19). Dasatinib is approved by the U.S. Food and Drug Administration (FDA) for patients with imatinib-resistant chronic myeloid leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia. Since inhibition of Src has the potential to inhibit the development and progression of metastases, Src inhibitors are being further investigated as anti-metastatic agents in both adjuvant and treatment settings (20, 21). Src signaling and the efficacy of SFK pathway inhibition has not been well studied in advanced thyroid cancer, and no studies have addressed the role of this pathway in thyroid cancer metastasis. In one previous study, FAK protein was overexpressed in a subset of PTC and ATC, but the phosphorylation status of FAK was not examined (22). We were the first to show that FAK is phosphorylated on a well characterized Src-dependent site (Y861) in a subset of PTC patient tumor samples, and we predict these tumors will be more aggressive and/or responsive to Src-directed therapies (23). The goals of this study were to evaluate the effects of Src inhibition using the clinically available Src inhibitor, dasatinib, on thyroid cancer growth and metastasis in vitro and in vivo.

Materials and Methods

Cell culture and generation of stable cell lines

Human thyroid cancer cell lines C643, TPC1, SW1736, BCPAP, K1, 8505C, HTh74, and HTh7 were authenticated by short tandem repeat profiling, as previously described (24). Cells were grown in RPMI (Invitrogen) containing 5%FBS (HyClone) and maintained at 37°C in 5% CO2 (24). BCPAP cells were transduced with pBABE-hygro, pBABE-WT-c-Src (Addgene plasmid 26983), or pBABE-c-Src-Dasatinib-Resistant-T338I (Addgene plasmid 26980) retrovirus, and selected with hygromycin (0.1 mg/ml) (25).
**Cellular growth assays**

Cells (500/well for TPC1 and K1, 1000/well for 8505C, 1500/well for C643, and 2000/well for BCPAP, SW1736, HTh74, and HTh7) were plated in triplicate in 96-well plates, and 24 hours later, cells were treated with increasing doses of dasatinib (BMS-354825; Bristol-Myers Squibb), as indicated. Cell growth was analyzed using the sulforhodamine B (SRB) assay (26). Briefly, after 72 hours of drug exposure, cells were fixed with 10% trichloroacetic acid at 4°C, and stained with 0.057% SRB (Sigma), after which unbound stain was removed by washing with 1% acetic acid. Protein-bound SRB was solubilized with 10 mmol/L unbuffered Tris base, and optical density was measured at absorbance wavelength of 570 nm.

**Cell cycle analysis**

Subconfluent cells were treated with vehicle (DMSO), 10, or 50 nM dasatinib in RPMI containing 5% FBS for 48 hours, as previously described (23, 27). Cell pellets were collected and stained with saponin/propidium iodide, and cell cycle distribution was determined as previously described (23, 27).

**Apoptosis assays**

Cells were plated in duplicate in white-walled 96-well plates, and 24 hours later treated with vehicle (DMSO), 10 or 50 nM dasatinib for 24 hours in media supplemented with 0.1% FBS. Caspase-3/7 activities were measured using a luminescent CaspaseGlo-3/7 Assay (Promega).

**Soft Agar Colony Formation**

Cells (10^4) were suspended in 0.3% agar with complete media, and plated on a base layer of 0.64% agar (Difco Agar Noble, BD Biosciences) in 6-well dishes. Cells were treated...
with vehicle (DMSO), 10, or 50nM dasatinib, and media containing vehicle or drug was replenished every 3-4 days for a total of 20 days. Colonies were stained with nitroblue tetrazolium chloride (Amresco; 5 mg/mL), and incubated overnight at 37° C to develop the stain. Colonies were counted using Image J software.

**Western blotting**

Cells were treated with the indicated doses of dasatinib or vehicle (DMSO) and harvested in CHAPS lysis buffer containing 10 mmol/L CHAPs, 50mmol/L Tris (pH 8.0), 150 mmol/L NaCl, and 2 mmol/L EDTA with 10 μmol/L Na₃VO₄ and 1x protease inhibitor cocktail (Roche). Protein extracts were resolved on 8% or 10% PAGE-SDS gels and transferred to Immobilon-P membranes (Millipore), as previously described (23, 27). Membranes were incubated at 4° C overnight with the following antibodies: pY416-SFK, SFK, Lyn, Fyn, Fgr, Yes, c-Src, pY710-Stat3, Stat3, pY410-130Cas, pY118-Paxillin, Paxillin, pS473-Akt, Akt, FAK (Cell Signaling), pY861-FAK (Invitrogen), ppERK1/2, total ERK2 (Santa Cruz), or α-tubulin (CalBiochem) diluted in 5% nonfat dry milk or 5% BSA in 20 mmol/L Tris, pH 7.4, 138 mmol/L NaCl, 0.1% Tween (TBST). Blots were incubated with secondary goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated antibodies (GE Healthcare) and proteins were detected by enhanced chemiluminescence (ECL) detection (Pierce) (23, 27).

**Immunoprecipitation**

Cells were lysed in RIPA buffer (10 mmol/L Tris (pH7.4), 100 mmol/L NaCl, 1 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.1%SDS, 10% glycerol, 0.5% sodoim deoxycholate, 1% Triton X-100, 1 mmol/L PMSF, 1x protease inhibitors, 2 mmol/L Na₃VO₄). Equal amounts of protein (500 μg) were incubated with 100 ng of c-Src or Lyn antibody (Cell Signaling) or control IgG overnight at 4° C with 15 μl of beads. Immunoprecipitates were washed with RIPA, resolved on
10% PAGE-SDS gels, and detected using the ImmunoCruz IP/WB optima F system (Santa Cruz) with the indicated primary antibodies and ECL as described above.

Orthotopic murine model

BCPAP and 8505C cells (5 x 10^5) engineered to express a luciferase-IRES-GFP plasmid were injected into the right thyroid lobe of nude mice, as previously described (28-30). Briefly, male athymic nude mice (NCI; ~30 grams; 10-12 weeks old) were anesthetized with tribromoethanol (250 mg/kg). Thyroid cancer cells (5 μL cell suspension) were injected into the right thyroid gland with the aid of a dissecting microscope (Nikon SMZ645), and the skin closed with staples. Tumor establishment and progression was measured weekly by detection of bioluminescence with the Xenogen IVIS200 system (Caliper) in the UCCC Small Animal Imaging Core. Briefly, mice were injected with D-luciferin (3 mg in 200 μL), and bioluminescence activity (photons/sec) was quantitated using the Living Image 2.60.1 software (Igor Corp). Min/max thresholds were normalized in order to compare images using the same scale. Final thyroid tumor size was measured with calipers and volume was calculated using the following formula: tumor volume = (length x width x height)/0.5236.

Experimental Metastasis Model

Male athymic nude mice (Harlan; 25 grams; 5 weeks old) received D-luciferin (3 mg) via IP injection, and five minutes later each mouse was anesthetized using isofluorane. BCPAP-luc-IRES-GFP cells (10^5 cells in 100 ul PBS) were injected into the left ventricle of nude mice using a 25-gauge needle, as previously described (31). Successful left ventricle injection was monitored by the pulsatile flow of red blood into the needle hub indicating correct placement, and by whole body bioluminescence immediately following injection. Metastatic progression was monitored weekly by IVIS imaging. Mice were sacrificed if they lost > 20% body weight,
and based on moribund criteria. All animal studies were performed in accordance with the animal protocol procedures approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver.

Drug preparation and administration

For in vivo studies, dasatinib (50 mg/kg) was prepared for daily oral gavage (5 days/week) in 80 mM sodium citrate buffer, pH 3.0. For the orthotopic murine model, mice were randomized on day 10 based on bioluminescence activity to receive drug or vehicle. In the metastatic murine model, mice received dasatinib or vehicle, as described above, starting 2 days before intracardiac injection (pre-treatment), or on day 11 following randomization (post-treatment).

Results

Effects of dasatinib on thyroid cancer cell growth, apoptosis, and transformation.

To study the role of SFK signaling in thyroid cancer, a panel of 8 thyroid cancer cell lines representing distinct thyroid tumor types (papillary thyroid cancer, PTC and anaplastic thyroid cancer, ATC), and clinically relevant oncogenic mutations (BRAF, RAS, RET/PTC1, PIK3CA) (Table 1 and (24)) was used to study sensitivity to dasatinib (32). Figure 1A shows that treatment with increasing doses of dasatinib (0.019 to 1.25 μM) for 3 days inhibited the growth of the C643, TPC1, BCPAP, and SW1736 cell lines by ~50% at low nanomolar concentrations, while higher concentrations were required to inhibit the growth of the K1 cell line. In contrast, the 8505C, HTh7, and HTh74 cell lines were not inhibited > 50% by dasatinib treatment, even at doses > 1 μM (Fig. 1A). IC50 values were calculated (Table 1), and cell lines exhibiting IC50 values of < 100 nM were considered sensitive to dasatinib, and include the TPC1, SW1736, BCPAP, and C643 cells with IC50 values between 30-80 nM. The K1 cell line exhibited
intermediate sensitivity (IC50=400 nM), and the remaining cell lines (8505C, HTh74, HTh7) were resistant to dasatinib with IC50 values > 1.6 μM (Table 1). The 100 nM “sensitive” demarcation was chosen based on previous studies demonstrating decreased selectivity of dasatinib at doses > 100 nM (32, 33). Overall, we observed similar sensitivity to inhibition of Src with dasatinib and saracatinib in our previous study (23), indicating an important role for SFKs in thyroid cancer cell growth.

To examine the mechanisms of inhibition of cell growth, cell cycle progression was evaluated in representative dasatinib-sensitive and dasatinib-intermediate cell lines (BCPAP, SW1736, K1), as well as dasatinib-resistant cell lines (8505C, HTh7, HTh74). Table 2 shows that treatment with 10 nM or 50 nM dasatinib resulted in a ~9-22% increase of cells in the G1 population in the BCPAP and SW1736 and K1 cells, and a corresponding 7-18% decrease in the percentage of cells in S phase. In contrast, dasatinib treatment of the resistant 8505C, HTh7, and HTh74 cell lines resulted in a 0-10% increase in cells in the G1 population (Table 2), consistent with the resistant nature of these cells (Fig. 1A). To further explore the mechanism of growth inhibition by dasatinib, induction of apoptosis was also tested. Figure 1B shows a higher induction of caspase-3/7 activity in the dasatinib-sensitive cell lines (BCPAP and SW1736; ~2-4 fold; p<0.05), whereas the dasatinib-intermediate (K1) and dasatinib-resistant (8505C) cells were less sensitive to dasatinib treatment, with a 1.2- to 2.5-fold induction of caspase-3/7 activity with the 10 and 50 nM doses, respectively. Overall, these data indicate that cell cycle and apoptosis may be important mechanisms mediating growth inhibition in the sensitive cell lines, but that different dominant mechanisms are present in different cell lines.

To determine whether Src inhibition with dasatinib affects anchorage independent growth, PTC and ATC cells exhibiting high (BCPAP), intermediate (K1), and low (8505C) sensitivity to dasatinib were tested. Figure 1C shows that treatment with 10 or 50 nM dasatinib
results in a dose-dependent decrease in colony formation in the dasatinib-sensitive BCPAP cells (36-78% inhibition; p<0.008), and dasatinib-intermediate K1 cells (49-85% inhibition; p<0.008). Colony formation of the 8505C cells, which were resistant to inhibition of growth with dasatinib (Fig. 1A; Table 2), were also not significantly inhibited under nonadherent conditions (Fig. 1C).

**Dasatinib inhibition of SFK and FAK signaling in thyroid cancer cells.** We next evaluated inhibition of activated SFKs and known downstream targets, in response to dasatinib treatment, in four PTC and ATC cell lines exhibiting high (BCPAP, SW1736), intermediate (K1), or low (8505C) sensitivity to dasatinib. Figure 2A shows that basal, endogenous SFK activity and expression is similar in all cell lines, treatment with dasatinib results in variable inhibition of SFK activity at the 1-10 nM doses, and complete inhibition at 50 nM dasatinib. Similar results were observed at earlier time points (4 hours, data not shown).

FAK phosphorylation on the well-characterized Src-dependent site, tyrosine residue 861 (Y861), was also evaluated. Figure 2A shows similar levels of basal pY861-FAK in all four cell lines, with lower levels in the K1 and 8505C cells. Similar to pY416-SFK, dasatinib treatment resulted in decreased, but variable, pY861-FAK levels at 1-10 nM doses of dasatinib, and complete inhibition at 50 nM dasatinib in all cell lines (Fig. 2A).

p130Cas and paxillin are key downstream targets of the Src-FAK complex that function as adaptor proteins to promote tumor cell survival, proliferation, and migration (34-36). We therefore evaluated levels of pY410-130Cas and pY118-paxillin, and inhibition by dasatinib. Figure 2A shows elevated levels of pY410-p130Cas and pY118-paxillin are present in PTC and ATC cells, and that treatment with increasing doses of dasatinib results in the inhibition of both p-p130Cas and p-paxillin. Taken together, these data indicate that dasatinib effectively inhibits
the Src-FAK-p130Cas-paxillin pathway in thyroid cancer cells, and that inhibition of this pathway does not distinguish sensitive from resistant thyroid cancer cells.

Effects of dasatinib treatment on ERK1/2, Stat3, and Akt signaling

Given the importance of the MAP kinase pathway in thyroid cancer, we evaluated the interaction of the Src pathway with MAP kinase in a panel of 4 thyroid cancer cell lines harboring oncogenic mutations in the MAP kinase pathway. Figure 2B shows that inhibition of Src with dasatinib does not inhibit MAP kinase (ppERK1/2) at low doses (1 or 10 nM), consistent with our previous studies using saracatinib (23). However, partial, but variable inhibition of ppERK1/2 was observed at higher doses of dasatinib (50 nM) (Fig. 2B and data not shown), suggesting that treatment with dasatinib may affect the oncogenic MAP kinase signaling in thyroid cancer.

We also evaluated the effects of SFK inhibition on the phosphorylation of Stat3 and pAkt signaling, which have been associated with resistance to Src inhibitors, and promote survival in other tumor models (37-40). Figure 2B shows that pStat3 was not detected in the PTC-derived BCPAP and K1 cell lines, while elevated levels were detected in the ATC-derived SW1736 and 8505C cells. Interestingly, no significant effects of dasatinib were observed on pStat3 levels. Fig. 2B shows that pAkt levels are low in BRAF-mutant thyroid cancer cells (Fig. 2B; BCPAP, SW1736, 8505C), while elevated levels of pAkt are present in the BRAF/PIK3CA-mutant K1 cells, where treatment with higher doses of dasatinib (50 nM) were required to inhibit pAkt (Fig. 2B). Taken together, these data indicate that Stat3 and Akt signaling are not major targets of Src in thyroid cancer.

Expression of Src Family Kinases in thyroid cancer cells. Since dasatinib has the potential to inhibit all Src family kinases (41), we evaluated expression of specific SFKs expressed in thyroid
cancer cells, which has not been previously characterized. Figure 3A shows that c-Src and Lyn are the only family members expressed, while Fyn, Yes, Lck, and Fgr were not detected. To determine whether c-Src and Lyn are activated in thyroid cancer cells, extracts were prepared from cells treated with or without dasatinib, and c-Src or Lyn were immunoprecipitated using anti-c-Src or anti-Lyn specific antibodies, followed by Western blotting to detect activation of c-Src and Lyn using the pY416Src antibody, which cross-reacts with the activated and autophosphorylated form of c-Src (pY416) and Lyn (pY397). Figure 3B (top) shows that equivalent levels of total c-Src are immunoprecipitated in BCPAP cells treated with or without dasatinib. Activated pY416Src is specifically detected in the c-Src immunoprecipitates, and pY416Src is inhibited by dasatinib treatment (Fig. 3B top). Lyn was also efficiently immunoprecipitated, but phosphorylation of Lyn was not detected in the Lyn immunoprecipitations (Fig. 3B). Similar results were observed in the 8505C cells (Fig. 3B, bottom). Taken together, these data indicate that c-Src is the predominant SFK activated in representative sensitive (BCPAP) and resistant (8505C) thyroid cancer cells.

In addition to its role as a potent SFK inhibitor, dasatinib is a multikinase inhibitor (32). To test the specific role of c-Src in response to dasatinib treatment, we tested the ability of a c-Src inhibitor-resistant transgene to rescue the effects of dasatinib on signaling and cell growth. For these studies we took advantage of the gatekeeper residue of Src (T338), which is required for dasatinib binding, to test whether expression of this dasatinib-resistant mutant rescues cells from dasatinib treatment (25, 42, 43). The c-Src dasatinib-resistant mutant (T338I, herein referred to as Src-RES), wild-type Src (Src-WT), or empty vector (pBABE-hygro) were stably expressed in the Src inhibitor-sensitive BCPAP cells at near physiologic levels (~1.5-fold above endogenous c-Src (Fig. 3C and data not shown). As expected, dasatinib treatment inhibits pY416SFK and pY861FAK in cells expressing empty vector (pBABE-hygro) or Src-WT (Fig. 3C). In cells expressing Src-RES, pY416SFK levels were not inhibited by dasatinib treatment,
indicating that the gatekeeper residue, Thr 338, is necessary for inhibition of Src by dasatinib.

Similarly, inhibition of pY861FAK by dasatinib was also blocked in cells expressing the Src-RES mutant, consistent with this residue being a major phosphorylation site targeted by Src (Fig. 3C).

We next tested the c-Src-dependent effects of dasatinib on growth of the BCPAP cells (Fig. 3D). As expected, we observed a dose dependent decrease of growth in Src-WT expressing cells, with an IC50 of 42 nM, consistent with the response of parental (nontransduced) and empty vector transduced cells (40 nM; Table 1). Notably, expression of the Src-RES gatekeeper mutant blocked the growth inhibitory effects of dasatinib, shifting the IC50 to 1200 nM (Fig. 3D). Similar results were observed in the SW1736 cells (data not shown). Thus, these data provide strong evidence that c-Src is the primary mediator of cell growth in response to Src inhibitor treatment in thyroid cancer cells.

Inhibition of Src with dasatinib inhibits PTC growth in a preclinical orthotopic mouse model.

We next evaluated the in vivo antitumor effects of Src inhibition in an orthotopic thyroid cancer model using the BCPAP cells (sensitive to dasatinib in vitro) and 8505C cells (resistant to dasatinib in vitro). BCPAP or 8505C cells stably expressing a luciferase-IRES-GFP plasmid were injected into the right thyroid gland of athymic nude mice, and tumor establishment and progression were monitored by measuring luciferase activity using the Intravital Imaging System (IVIS). Daily treatment with dasatinib (50 mg/kg) was initiated on day 10. Using this approach, a significant inhibition of BCPAP orthotopic tumor growth was observed 6 days post-treatment (day 16, p=0.014), which was sustained through days 23 and 29 (p= 0.0003), compared to vehicle-treated mice (Fig. 4A, left). Representative bioluminescence images of orthotopic tumors are shown in Figure 4B. The BCPAP orthotopic final tumor volumes were inhibited by >90% in response to dasatinib treatment (p=0.0014; mean vehicle tumor volume = 289 mm³;
mean dasatinib tumor volume = 11 mm³; Fig. 4C left). Strikingly, three out of eight dasatinib-treated BCPAP orthotopic mice did not have measurable tumor after therapy. In contrast, the growth of 8505C-derived tumors was not significantly inhibited by dasatinib treatment at any time point, as measured by bioluminescence imaging (Fig. 4A, right), and final tumor volumes (p=0.8; Fig. 4C, right). Thus, these data validate our in vitro findings and show that inhibition of Src with dasatinib strongly inhibits thyroid tumor growth in vivo.

Inhibition of Src with dasatinib blocks PTC metastasis.

One of the most lethal characteristics of thyroid cancer is the metastatic spread to distant sites, especially to the bone. The orthotopic model has resulted in limited distant metastases, and to date, there have been no bone metastasis models in thyroid cancer. We have therefore established an experimental metastasis model using an intracardiac injection approach which allows for the widespread dissemination of tumor cells. Using this model system, we tested the Src inhibitor-sensitive BCPAP (PTC) cells, which have formed consistent distant metastases with a 70-90% take rate. For these studies, BCPAP-luc-IRES-GFP cells were injected into the left ventricle of athymic nude mice. Successful left ventricle injection was monitored by the spontaneous entrance of pulsatile blood into the hub of the syringe, and by bioluminescence imaging immediately after injection to visualize the widespread distribution of luciferase expressing tumor cells (Fig. 5A). Representative histologic images of bone metastases are shown in Fig. S1.

To determine the role of Src in metastasis, we first utilized an adjuvant treatment approach, in which mice were given vehicle or dasatinib (50 mg/kg) by daily oral gavage 48 hours before intracardiac injection of cancer cells. Using this approach, we observed a ~70% take rate in the vehicle-treated mice, compared to the pretreatment group where none of the mice developed distinct metastatic tumors, even after 6 weeks (Fig. 5C). Representative
bioluminescence images of metastatic tumors are shown in Fig. 5B. Treatment with dasatinib was stopped on day 56, and Figure 5D shows that dasatinib-treated mice had a significantly increased overall survival when compared to vehicle treated mice, with a mean survival of 63 days for the vehicle-treated mice versus 123 days for the dasatinib-treated mice (Fig. 5D; p = 0.0015). Next, to determine the effects of dasatinib treatment on established metastases, these studies were repeated and mice were treated with dasatinib (50 mg/kg) after the establishment of metastases on day 11 (Fig. S2A). Figure S2C shows that dasatinib treatment resulted in a sustained inhibition of metastatic tumor growth starting ~10 days after treatment (2-way ANOVA; p = 0.004). Representative images, showing inhibition of metastasis at week 7, are shown (Fig. S2B). Overall, these results provide the first demonstration for Src signaling in thyroid cancer metastasis, and indicate that inhibition of Src with dasatinib represents a promising therapeutic strategy to block both primary and metastatic tumor growth in thyroid cancer patients.

**Discussion**

Little is known about the role of Src and FAK and the efficacy of SFK inhibitors in thyroid cancer. Our group was the first to demonstrate that elevated levels of phospho-SFK and FAK are present in thyroid cancer cell lines, and that phospho-FAK is present in a subset of PTC tumor samples (23). We further showed that inhibition of Src with the more selective Src inhibitor, saracatinib, inhibits the growth and invasion of thyroid cancer cell lines expressing elevated levels of phospho-FAK (23). In the current study, we have further tested the role of SFK signaling in thyroid cancer using dasatinib, which is a potent ATP-competitive inhibitor of Src, which is FDA-approved for the treatment of patients with leukemia, and is being tested in phase II clinical trials for several solid tumors (20). Here, we show that Src inhibition with dasatinib results in the inhibition of thyroid cancer cell growth and transformation *in vitro*, as well
as primary and metastatic tumor progression \textit{in vivo}, providing strong rationale for the use of Src inhibitors in patients with advanced thyroid cancer.

In this study, we identified cells with high, moderate, and low sensitivity to dasatinib \textit{in vitro}, and found that treatment with dasatinib blocks SFK and FAK signaling. Interestingly, we did not find a clear correlation between basal levels of these signaling molecules, or inhibition of these molecules by dasatinib with sensitivity to dasatinib, although phospho-FAK levels may be higher in the sensitive cell lines (Fig. 2A). The lack of correlation between phospho-SFK levels and sensitivity to dasatinib is similar to our previous studies using the Src inhibitor, saracatinib, as well studies in other tumor types, and suggest that even low levels of SFK activity are sufficient for these biological responses (23, 44, 45). We also evaluated Stat3 and Akt signaling, which have been shown to promote survival and mediate resistance to Src inhibitor treatment in other tumor types (37-40). Importantly, we did not observe regulation of pStat3 in response to dasatinib treatment, and although pAkt was inhibited with higher concentrations of dasatinib in the \textit{BRAF/PIK3CA}-mutant K1 cells, low to undetectable levels of pStat3 and pAkt were observed in both sensitive and resistant cell lines, indicating that these pro-survival pathways are likely not major targets of Src signaling in thyroid cancer. Finally, we found that dasatinib treatment inhibited ppERK1/2 at higher concentrations, but that this inhibition was variable, suggesting that the MAP kinase pathway is not a major target of Src in thyroid cancer cells. Accordingly, we did not identify any correlation between known oncogene mutations (\textit{BRAF, RAS, PIK3CA, RET/PTC1}) and sensitivity to dasatinib (Table 1), consistent with our previous studies with the more selective Src inhibitor, saracatinib (23), as well as other tumor types (33, 40). Thus, additional studies are needed to identify predictive biomarkers of response to determine which patients will benefit the most from Src-directed therapies.
Although dasatinib is a multi-kinase inhibitor, in addition to a potent inhibitor of Src, our data show a strong correlation between the sensitivity of thyroid cancer cells to dasatinib (Fig. 1) and the more selective Src inhibitor, saracatinib (23), indicating an important role for Src in thyroid cancer cell growth, similar to one previous study in thyroid cancer (46). We have further shown the inhibitory effects of dasatinib are accompanied by inhibition of the Src pathway at low nanomolar concentrations (Fig. 2A), indicating these effects are likely mediated by Src and FAK signaling. To identify the specific SFK important in thyroid cancer, we characterized the expression of six SFK members in thyroid cancer cells, and show that c-Src and Lyn are the predominant members expressed (Fig. 3A). Interestingly, we found that while both c-Src and Lyn are expressed, c-Src is the predominant SFK phosphorylated (Fig. 3B). Since dasatinib can inhibit all Src family kinase members, as well as other kinases, we further tested the role of c-Src in the response to dasatinib using a gatekeeper approach. These studies showed expression of the c-Src gatekeeper mutant (Src-RES) rescues dasatinib-mediated inhibition of thyroid cancer cell growth, as well as inhibition of Src signaling, providing strong evidence that c-Src is the primary mediator of growth in response to dasatinib treatment (Fig. 3D). While these results do not prove Lyn has no effect, these data strongly support c-Src as the dominant if not only mediator of growth in response to Src inhibitors in thyroid cancer cells. These results are in contrast to recent studies in prostate and breast cancer, where both c-Src and Lyn are expressed and activated, and shown to mediate distinct biologic responses (15, 16, 47).

A number of studies in other tumor types, including colon cancer and NSCLC have shown that Src primarily regulates invasion and metastasis (33, 48, 49). Our studies show that treatment with dasatinib not only blocks transformation and metastatic progression, but also inhibits thyroid cancer cell growth. These results indicate that the use of Src inhibitors may have higher therapeutic efficacy in thyroid cancer, due to the potential to inhibit primary tumor growth (Fig. 4), in addition to metastatic outgrowth (Fig. 5; Fig. S2).
Distant metastasis to the bone represents one of the most lethal characteristics of thyroid cancer, and there are currently no effective therapeutic options for these patients. Due to the role of Src signaling in bone metastases in other tumor types, and the importance of distant metastases in thyroid cancer, we developed an experimental metastasis model using an intracardiac injection approach. This is the first report describing the establishment of a thyroid cancer metastasis model to bone. Using a pre-treatment, adjuvant approach, we found that while ~70% of mice developed metastases in the vehicle-treated control group, no mice developed metastases in the dasatinib-pretreated group after 6 weeks of treatment (Fig. 5). We found that although these mice exhibited a significantly longer survival of ~60 days compared to vehicle-treated mice, even after discontinuation of dasatinib (Fig. 5D), the majority of these mice developed tumors. To determine if inhibition of Src could block the progression of established metastases, we used a post-treatment approach, where mice were treated with dasatinib after metastatic tumor establishment (Fig. S2). Using this approach, dasatinib treatment significantly inhibited metastatic tumor progression by ~10-fold (Fig. S2). Taken together, these results indicate that Src activity is important for tumor cell growth and/or survival, rather than early metastatic cell seeding. These results are consistent with our in vitro growth data (Fig. 1), as well as previous studies in prostate and breast cancer (16, 25). Thus, the precise role of Src in the progression of latent metastases, which is an important clinical problem in thyroid cancer (50), will be of particular interest to test in future studies. Collectively, these studies indicate that Src inhibitors will likely need to be used long-term or in combination with other therapies in order to achieve complete tumor remission, but nonetheless, these data provide a strong rationale for Src inhibitors as anti-tumor and anti-metastatic agents in patients with advanced, metastatic disease.

In conclusion, our results show that inhibition of Src with dasatinib inhibits thyroid cancer cell growth and transformation in vitro and tumor growth and metastatic outgrowth in vivo. We
show for the first time that c-Src and Lyn are the predominant SFK members expressed, and that c-Src is likely the major target of dasatinib in thyroid cancer cells. Finally, we provide the first evidence that dasatinib inhibits the progression of thyroid cancer metastasis \textit{in vivo}. Thus, along with the growth inhibition observed in our orthotopic model, these results provide strong justification for the use of Src inhibitors as both anti-tumor and anti-metastatic agents for advanced thyroid cancer patients.
Acknowledgements

We thank Drs. Jeffrey Myers and Maria Gule at MD Anderson Cancer Center for their guidance in establishing the orthotopic thyroid cancer model, and Dr. Carol Sartorius for her guidance in establishing the intracardiac injection model. We thank Dr. Arthur Gutierrez-Hartmann for advice and critical reading of the manuscript, Dr. Jena French for histology expertise, and Dalan Jensen for statistical support. We also thank Bristol-Myers Squibb for generously providing dasatinib for these studies.

Grant support

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References

Figure Legends

Figure 1. Effects of dasatinib on thyroid cancer growth, survival, and transformation. A) Thyroid cancer cells were treated with the indicated doses of dasatinib or vehicle for 3 days and growth was measured using the sulforhodamine (SRB) assay (26). Growth of the vehicle-treated cell was set to 100% (not shown). Results are the mean % inhibition compared to vehicle-treated cells +/- SEM of three experiments performed in triplicate. The dashed line represents 50% growth inhibition. Significant inhibition of growth by one-way ANOVA was observed in the C643, TPC1, BCPAP, SW1736 (p < 0.0001), K1 (p = 0.001), and HTh7 (p = 0.01), although > 50% growth inhibition was not observed in the HTh7 cells. B) Cells were plated in RPMI containing 0.1% FBS with or without the indicated dose of dasatinib or vehicle for 24 hours, and Caspase 3/7 activity was determined using the Caspase-3/7 Glo assay, as described in Materials and Methods. Caspase 3/7 activity of the vehicle-treated cells was set to 1 and is represented by the dashed line. Results are the mean +/- SEM of three experiments performed in duplicate (*p < 0.05). C) BCPAP, K1, and 8505C cells were plated in soft agar, and one day later, cells were treated with 10 or 50 nM dasatinib or vehicle. Media with or without drug was replaced every 3-4 days for a total of 20 days. Number of colonies was counted using the Image J software. Colony formation of the vehicle-treated cells was set to 100% and is indicated by the dashed line. Percent colony formation of the dasatinib-treated cells compared to vehicle is shown. Results are the mean +/- SEM of three experiments performed in duplicate (* p < 0.008).

Figure 2. Effects of Src inhibition with dasatinib on cell signaling. Cells were treated with the indicated doses of dasatinib or vehicle for 24 hours. Cells were lysed in CHAPS buffer and equal protein (30 μg) was analyzed by Western blotting on 8% PAGE-SDS gels with the indicated antibodies. Blots shown are representative of at least three separate experiments.
Figure 3. Src Family Kinase Expression in Thyroid Cancer Cells and Effects of a Src Gatekeeper Mutant. A) Expression of the indicated SFKs was measured by Western blot analysis in thyroid cancer cells (30 μg extract each), as described in Fig. 2. The following positive control extracts (10 μg each) were loaded for the indicated antibody: Jurkat (c-Src, Fyn, Lck), Raji (Lyn, Fgr), and U87 MG (Yes). α-tubulin was used as a loading control. B) Cells were treated with or without dasatinib (50 nM) for 4 hours and cell extracts (500 μg) were immunoprecipitated with anti-c-Src, anti-Lyn, or rabbit IgG. Immunoprecipitates were analyzed by Western blot analysis with the indicated antibodies. The pY416SFK antibody cross-reacts with the activation site of c-Src (Y416) and Lyn (Y397). Results shown are representative blots of at least three experiments. C) BCPAP cells were transduced with retroviruses expressing wild-type Src (Src-WT), the T338I gatekeeper mutant Src (Src-RES), or empty vector (pBABE-hygro), and selected with hygromycin for 7 days prior to experimental manipulation. Cells were treated with dasatinib (50 nM) or vehicle (DMSO) for 24 hours and cell extracts (30 μg) were analyzed by Western blot analysis with the indicated antibodies. D) BCPAP cells expressing Src-WT or Src-RES were plated in 96 wells. The following day, cells were treated with the indicated concentrations of dasatinib or vehicle (DMSO) for 3 days and growth was measured using the SRB assay as described above. The growth of the vehicle-treated cells was set to 100% (not shown). Results shown are the mean % inhibition compared to vehicle-treated cells, +/- SD from three experiments performed in triplicate. Inhibition of growth by 50% is represented by the dashed line.

Figure 4. Inhibition of thyroid cancer growth by dasatinib in an orthotopic mouse model. A) BCPAP-luc PTC or 8505C-luc ATC cells (5 x 10^5) were injected into the right thyroid gland of nude mice and tumor establishment and growth were monitored by IVIS imaging. Dosing by daily oral gavage with dasatinib (50 mg/kg) or vehicle was started on day 10 and continued for
the duration of the experiment. Data are expressed as the mean +/- SEM area flux (photons/second) of the neck region at the indicated time points. (* p = 0.01; **p = 0.0003). B) Representative bioluminescence images are shown for each time point. The arrows indicate vehicle (blue) or dasatinib (red) treatment, which started at day 10. C) Final tumor volume (mm$^3$) on day 29 is shown. Data points represent tumor volumes of individual animals, and horizontal bars represent the mean tumor volume +/- SEM (n=7-8 animals per group for the BCPAP cells and 7-10 animals per group for the 8505C cells). BCPAP mean vehicle tumor volume = 289 mm$^3$; BCPAP mean dasatinib tumor volume = 11 mm$^3$. 8505C mean vehicle tumor volume = 98 mm$^3$; 8505C mean dasatinib tumor volume = 94 mm$^3$. Statistical analysis was performed using the Student’s $t$-test.

**Figure 5. Inhibition of metastasis by adjuvant dasatinib treatment in an experimental metastasis model.** Mice were pretreated with dasatinib (50 mg/kg) or vehicle by daily oral gavage two days before intracardiac (IC) injection of BCPAP cells (1 x 10$^5$/100 μl) into the left ventricle of athymic nude mice. A) Representative images of vehicle or dasatinib-treated mice obtained by IVIS imaging 1 minute after IC injection demonstrates widespread distribution of cancer cells. B) Representative images of metastases 6 weeks post-IC injection. C) Total bioluminescence activity (photons/sec) at week 6 is shown for the vehicle and dasatinib-pretreated mice. Data points represent whole body bioluminescence activity of individual animals. Horizontal bars represent the mean +/- SEM, n=9-13 per group. Statistical analysis was performed using the Student’s $t$-test. D) Kaplan-Meier curve representing overall survival of vehicle versus dasatinib-pretreated mice. Dasatinib treatment was stopped at day 56, and the survival experiment was stopped at day 130. Statistical analysis was performed using the log-rank test.
Figure 1

A. Graph showing the percentage growth of different cancer cell lines (C643, TPC1, BCPAP, SW1736, K1, 8505C, HTh7, HTh74) in response to varying concentrations of Dasatinib (μM).

B. Bar graph showing the fold change in colony formation of BCPAP, SW1736, K1, and 8505C cell lines at 10 nM and 50 nM Dasatinib.

C. Bar graph showing the percentage colony formation of BCPAP, K1, and 8505C cell lines at 10 nM and 50 nM Dasatinib.

* indicates statistical significance.
Chan et al Figure 2

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- pY416-SFK
- SFK
- pY861-FAK
- FAK
- pY410-p130Cas
- p130Cas
- pY118-paxillin
- α-tubulin

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**8505C**

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**D**

![Graph showing growth inhibition with dasatinib for WT Src and Src-RES](image)

- WT Src
- Src-RES

Dasatinib (μM): 0.019, 0.038, 0.075, 0.15, 0.3, 0.625, 1.25
A

BCPAP

8505C

photons/sec (x 10^8)

photons/sec (x 10^9)

Vehicle

Dasatinib

Vehicle

Dasatinib

Tx day 10

Day

Day

B

BCPAP

8505C

Day 7

Day 16

Day 23

Day 29

Day 23

Day 29

Vehicle

Dasatinib

Vehicle

Dasatinib

C

BCPAP

8505C

Final Tumor Volume (mm^3)

Final Tumor Volume (mm^3)

Vehicle

Dasatinib

Vehicle

Dasatinib

p = 0.0014

p = 0.8
Figure 5

A

Control

Dasatinib Pre-treat

1 min image after IC injection

B

Ventral

Dorsal

Week 6

C

Bioluminescence (p/s)

4 x 10^9

2 x 10^9

1 x 10^7

7.5 x 10^6

5 x 10^6

2.5 x 10^6

0

Vehicle

Dasatinib

p = 0.016

D

Percent survival

100

80

60

40

20

0

Days

0 10 45 60 75 90 105 120

Control

Dasatinib pretreat

p = 0.0015

Dasatinib treatment stopped

p = 0.016

Vehicle

Dasatinib

p = 0.0015

Dasatinib treatment stopped
Table 1. Antiproliferative effects of the Src inhibitor, dasatinib. Thyroid cancer cells were treated with increasing doses of dasatinib for 3 days, as described in Fig. 1. IC50 values were calculated using nonlinear regression analysis with a slope sigmoidal dose response curve in GraphPad Prism. No highlighting and dark grey highlighting indicates sensitive and resistant cell lines, respectively, and light grey highlighting indicates intermediate sensitivity.

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Table 2. Effects of the Src inhibitor, dasatinib, on the cell cycle. Cells were treated with the indicated dose of dasatinib or vehicle for 48 hours. Cell cycle distribution was determined using the PI/saponin method. Results are the mean of 2-4 experiments.

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Targeted inhibition of Src kinase with dasatinib blocks thyroid cancer growth and metastasis

Christine M. Chan, Xia Jing, Laura A. Pike, et al.

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