Identification of potential biomarkers for measuring inhibition of Src kinase activity in colon cancer cells following treatment with dasatinib

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Abstract

Elevated levels of Src kinase expression have been found in a variety of human epithelial cancers. Most notably in colon cancer, elevated Src expression correlates with malignant potential and is also associated with metastatic disease. Dasatinib (BMS-354825) is a novel, orally active, multi-targeted kinase inhibitor that targets Src family kinases and is currently under clinical evaluation for the treatment of solid tumors. However, the effects of dasatinib on epithelial tumors are not fully understood. We show that concentrations of dasatinib that inhibit Src activity do not inhibit proliferation in 10 of 12 colon cancer cell lines. However, inhibition of integrin-dependent adhesion and migration by dasatinib correlated with inhibition of Src activity, suggesting that dasatinib may have anti-invasive or anti-metastatic activity and antiproliferative activity in epithelial tumors. Using phospho-specific antibodies, we show that inhibition of Src activity in colon cancer cell lines correlates with reduced phosphorylation of focal adhesion kinase and paxillin on specific Src-dependant phosphorylation sites. We have validated the use of phospho-specific antibodies against Src Tyr⁴¹⁹ and paxillin Tyrⁱ¹⁸ as biomarkers of dasatinib activity in vivo. Colon carcinoma—bearing mice treated with dasatinib showed a decrease in both phospho-Src Tyr⁴¹⁹ and phospho-paxillin Tyrⁱ¹⁸ in peripheral blood mononuclear cells, which correlated with inhibition of Src activity in the colon tumors. Thus, peripheral blood mononuclear cells may provide a useful surrogate tissue for biomarker studies with dasatinib using inhibition of Src Tyr⁴¹⁹ and paxillin Tyrⁱ¹⁸ phosphorylation as read-outs of Src activity. [Mol Cancer Ther 2006;5(12):3014–22]

Introduction

Src family kinases are involved in many aspects of tumor cell behavior, such as proliferation, survival, angiogenesis, adhesion, invasion, and metastasis (1–5). Src expression is frequently elevated in a number of epithelial tumors, including colon, breast, pancreas, lung, and ovarian, compared with the adjacent normal tissues (2, 3, 5). In colon cancer, increased Src expression is linked to malignant potential with increases seen in premalignant lesions and adenomas but with highest levels seen in malignant polyps (6–8). However, further increases in Src expression are also seen in metastatic tissues, and activating mutations have been found in a small subset of metastatic colon tumors, suggesting an additional role for Src in the metastatic progression of colon tumors (9–11). More recently, increased Src kinase activity in colon tumors was identified as an independent indicator of poor clinical prognosis in all stages of human colon cancer (12).

Much interest has, therefore, evolved around the development of Src kinase inhibitors for the treatment of cancer (13, 14). Dasatinib (BMS-354825) was identified as a highly potent, ATP-competitive inhibitor of Src and Abl kinases with antiproliferative activity in both hematologic and solid tumor cell lines (15). The presence of the Philadelphia chromosome in chronic myeloid leukemia results in a constitutively active Bcr-Abl kinase, which drives the pathogenesis of chronic myeloid leukemia, and the successful use of the Abl kinase inhibitor imatinib (Gleevec) in this disease has validated the use of tyrosine kinase inhibitors for the treatment of cancer. Dasatinib can inhibit the kinase activity of Bcr-Abl mutants found in chronic myeloid leukemia patients with acquired resistance to imatinib (16) and has promising activity in phase I/II clinical evaluation in patients with imatinib-resistant chronic myeloid leukemia (17, 18). Dasatinib also inhibits Src kinase activity in epithelial cell lines (19, 20) and is currently in phase I trials for the treatment of solid tumors (21). However, the potential effect of dasatinib in solid tumors may be multiple as effects on migration and invasion have been reported as well as inhibition of proliferation (15, 19, 20), and it remains unclear which of these mechanisms will become more relevant in the clinical application of dasatinib in solid tumors of epithelial origin.

The introduction of molecularly targeted agents into the clinic has led to a re-evaluation of clinical trial design as many of these agents are not cytotoxic, and conventional...
measures of reduction in tumor bulk and the use of maximum tolerated dose based on toxicity do not apply. This has brought to our attention the need for robust biomarkers of the biological activity of these agents in tumor cells to aid in establishing optimal therapeutic doses. Phospho-specific antibodies have provided useful reagents for analysis of signaling pathways in clinical samples. For example, in clinical trials of the epidermal growth factor receptor inhibitor gefitinib, phospho-specific antibodies raised against the activated epidermal growth factor receptor and the downstream mitogen-activated protein kinase were used to select optimal doses (22). As tumor biopsies are not readily available for these pharmacodynamic studies, the use of validated markers in surrogate tissues is often required.

In this study, we have identified autophosphorylation of Src on Tyr419 and phosphorylation of paxillin on Tyr185 as potential biomarkers of dasatinib activity in tumors and show for the first time that inhibition of Src kinase activity in peripheral blood correlates with effects in epithelial tumors. Furthermore, we show that concentrations of dasatinib that inhibit Src activity have no effect on the proliferation of 10 of 12 colon cancer cell lines but do inhibit cell adhesion and migration, suggesting that in addition to effects on tumor proliferation, dasatinib may also be useful as an anti-invasive and anti-metastatic agent.

Materials and Methods

Cell Culture and Drug Treatment

Colon cancer cell lines and SYF+/− cells were obtained from the American Type Tissue Collection (LGC Promochem, Teddington, United Kingdom) except for BE (a kind gift from N. Gibson, University of Southern California, Los Angeles, CA) and KM12C (a kind gift from I. Fidler, University of Texas M.D. Anderson Center, Houston, TX). Dasatinib (Bristol Myers Squibb, Princeton, NJ) was prepared as a 20 mmol/L stock in DMSO and diluted in culture media before treatment for the indicated times.

Cell Proliferation Assay

Cells were plated at an appropriate density in 96-well plates and after 24 hours were treated with a range of dasatinib concentrations. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay (23) was carried out after a further 72 h. Mean values were determined from quadruplicate wells and plotted on log-dose response curves as the mean percentage of the untreated controls. IC50 values were then calculated using XLFit software. The highest concentration of DMSO (drug diluent) added to the cells had no effect on cell proliferation.

Western Blot Analysis

Cells were washed and then lysed in 50 mmol/L Tris (pH 7.6), 150 mmol/L sodium chloride, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 μg/mL aprotinin, 125 mmol/L phenylmethylsulfonyl fluoride, 100 μmol/L sodium orthovanadate, and 0.5 mmol/L sodium fluoride. Cleared lysates were resolved by 4% to 12% Bis-Tris gel electrophoresis (Invitrogen, Paisley, United Kingdom); proteins were transferred to nitrocellulose then blocked and probed with either 1:1,000 anti-Src (Cancer Research UK), 1:10,000 anti-paxillin, 1:1,000 anti–focal adhesion kinase (anti-FAK; both BD Pharmingen, Oxford, United Kingdom), 1:1,000 anti–phospho-Src Tyr419, 1:1,000 anti–phospho-paxillin Tyr185 or 1:1,000 anti–phospho-FAK Tyr761 (all Biosource, Nivelles, Belgium). Bound antibody was detected by incubation with anti-mouse or anti-rabbit horseradish peroxidase–conjugated secondary antibody and visualized by enhanced chemiluminescence (Amersham, Little Chalfont, United Kingdom). Where indicated, the nitrocellulose was incubated with Re-Blot Plus (Chemicon, Chandlers Ford, United Kingdom) to remove bound antibody then reprobed with additional antibodies.

Cell Adhesion Assay

Black-walled, clear-bottomed, 96-well plates (Corning Costar, High Wycombe, United Kingdom) were coated with 1:250 dilution of Matrigel (BD Biosciences, Oxford, United Kingdom) or 10 μg/mL poly-l-lysine (Sigma, Dorset, United Kingdom) for 1 h at 37°C and then washed twice with PBS before use. Cells were trypsinized, washed with PBS, then incubated with calcein AM (Invitrogen) at a concentration of 5 μmol/L in normal growth medium ± dasatinib for 1 h at 37°C, with agitation every 15 min. Cells were then washed with PBS and resuspended in serum-free medium ± dasatinib. Cells (2 × 105) were added to each coated well of the 96-well plate and allowed to adhere for 40 min at 37°C. The plate was then washed twice with PBS, and fluorescence was measured at an excitation wavelength of 494 nm and an emission wavelength of 517 nm. Values represent the mean of quadruplicate wells ±SE and corrected for background autofluorescence as determined from coated wells containing serum-free medium.

Cell Migration Assay

Cells were plated at 5 × 105 per well of a six-well plate in DMEM containing 10% fetal bovine serum. After 24 h, confluent monolayers were scored with a fine pipette tip to produce a denuded area or wound. Migration into the wound was monitored by time-lapse video microscopy over 18 h in the presence or absence of dasatinib at ×20 magnification on a Zeiss Axiovert S100 microscope using AQM Advance software (Kinetic Imaging, Nottingham, United Kingdom). Three representative areas were scored for each treatment and the distance moved calculated using Tracking Analysis software (Kinetic Imaging).

Isolation of Peripheral Blood Mononuclear Cells

Human or mouse whole blood was collected into Becton Dickinson Vacutainer Cell Preparation Tubes (BD Biosciences, Oxford, United Kingdom) and centrifuged for 30 min at 1,700 × g. Peripheral blood mononuclear cells (PBMC) were removed from the resulting gradient and washed twice with PBS for 15 min at 300 × g. Cells were then washed in PBS for 5 min at 1,600 rpm and resuspended in cell extraction buffer (Biosource) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail II (both Sigma) at a dilution of 1:100. Lysis was
allowed to proceed for 30 min on ice, and then samples were centrifuged for 15 min at 13,500 rpm at 4°C. Cell lysates were snap frozen on dry ice and stored at −80°C until further processing.

**In vivo Analysis**

KM12C cells expressing activated Src (Src527F; ref. 24) were injected s.c. into the right flank of 4- to 6-week-old female CD31 nude mice (Charles Rivers, Harlan, United Kingdom). When the tumors were established, around 10 days after implantation, dasatinib was given by oral gavage in 80 mmol/L citrate buffer, which was also used as vehicle control. The mice were sacrificed 2 h later, and the tumors were excised, and blood was collected from eight mice. PBMCs were isolated as described above, and the tumors from each mouse were formalin fixed and paraffin embedded before immunohistochemical analysis using DakoCytomation Envision kit (DakoCytomation Ltd., Ely, United Kingdom). In brief, tumors were deparaffinized, rehydrated, and labeled with 1:100 anti–phospho-Src Tyr419 antibody (Calbiochem, Merck Biosciences Ltd., Nottingham, United Kingdom) or 1:100 anti–phospho-paxillin Tyr118 (Biosource) antibodies for 1 h.

**Tumor Microarray Analysis**

The MaxArray human colon carcinoma tissue microarray (Zymed, Invitrogen) was used. Each slide contains 60 tissue cores from different colon carcinomas. They consist of 49 adenocarcinomas, 7 mucinous carcinomas, 3 signet ring adenocarcinomas, and 1 adenouscarcinoma. The slides were stained as described above with 1:100 anti–phospho-Src Tyr419 or 1:250 anti–Src 36D10 antibodies (Cell Signaling, New England Biolabs, Hitchin, United Kingdom) for 18 h. Expression was scored as staining intensity using a three-point system and was carried out by three independent assessors. Only 7 of the 60 samples were given different scores, and in these cases, the samples were reassessed by each of the scorers, and a representative score agreed.

**Results**

**Inhibition of Tumor Cell Proliferation by Dasatinib**

**Does Not Correlate with Inhibition of Src Activity**

Inhibition of Src activity has been reported to inhibit the proliferation of some, but not all, tumor cell lines. We looked at the ability of dasatinib to inhibit proliferation in a panel of 12 colon carcinoma cell lines. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assays were carried out after exposure to a range of dasatinib concentrations for 72 h, and IC50 values were calculated (Table 1). Nine of the cell lines had IC50 values between 1 and 10 μmol/L, with WiDr and HT29 cells being the most sensitive with IC50 values of 0.03 and 0.05 μmol/L, respectively, whereas SW620 cells were the most resistant with an IC50 value of 16.41 μmol/L. The sensitivity of the cell lines to dasatinib did not correlate with Src protein levels as all the WiDr, HT29, and SW620 cells expressed equivalent levels of Src (Fig. 1A). Using Src autophosphorylation on Tyr419 as a measure of Src activity in cells, dasatinib treatment resulted in a dose-dependent inhibition of Src activity in all cell lines analyzed (Fig. 1B, shown here in SW620, HT29, H630, and WiDr cells). Inhibition of Src activity correlated with inhibition of proliferation in the WiDr and HT29 cells but not in the SW620 or H630 cells where much higher concentrations of dasatinib were required to prevent proliferation than was required for inhibition of Src activity. Furthermore, complete inhibition of Src activity was seen at 200 nmol/L dasatinib in SW620 cells over 72 h, which corresponded to the treatment period of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assays (Fig. 1C). This concentration of dasatinib had no effect on the proliferation of the SW620 cells. Thus, the ability of dasatinib to inhibit the proliferation of colon cancer cell lines seems to be largely independent of its ability to inhibit Src kinase activity, although there are some cell lines (e.g., WiDr and HT29) whose growth is inhibited by dasatinib at concentrations that inhibit Src kinase activity.

To further explore the ability of dasatinib to inhibit cell proliferation, experiments were carried out in fibroblasts lacking all three ubiquitous Src family members (SYF−/− cells; ref. 25). Over 5 days, the growth of the SYF−/− cells was inhibited by around 45% in cells treated with 100 nmol/L dasatinib (Fig. 1D). As these cells lack Src family kinases, the effects of dasatinib on their proliferation cannot be attributed to inhibition of Src activity. It is likely therefore that the ability of dasatinib to inhibit other kinases, such as the platelet-derived growth factor receptor and Abl, may also contribute to its effects on cell proliferation.

**Inhibition of Cell Adhesion and Migration by Dasatinib**

Correlates with Inhibition of Src Activity

Ssrc is known to regulate cell-matrix adhesion and migration of tumor cells. Using two colon cancer cell lines, we assessed the ability of dasatinib to block integrin-dependent adhesion to the reconstituted basement membrane Matrigel. There was a dose-dependent inhibition of HT29 and H630 cell adhesion to Matrigel (Fig. 2A), whereas adhesion to the non-integrin ligand poly-l-lysine was not

<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
<th>IC50* (μmol/L)</th>
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<tbody>
<tr>
<td>KM12C</td>
<td>Primary</td>
<td>3.22 ± 0.201</td>
</tr>
<tr>
<td>HT29</td>
<td>Primary</td>
<td>0.05 ± 0.004</td>
</tr>
<tr>
<td>H630</td>
<td>Liver metastasis</td>
<td>4.40 ± 0.613</td>
</tr>
<tr>
<td>COLO 320DM</td>
<td>Primary</td>
<td>10.39 ± 0.928</td>
</tr>
<tr>
<td>BE</td>
<td>Primary</td>
<td>1.58 ± 0.236</td>
</tr>
<tr>
<td>DLD-1</td>
<td>Primary</td>
<td>1.80 ± 0.198</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Primary</td>
<td>6.23 ± 0.283</td>
</tr>
<tr>
<td>WiDr</td>
<td>Primary</td>
<td>0.03 ± 0.002</td>
</tr>
<tr>
<td>SW620</td>
<td>Lymph node metastasis</td>
<td>16.41 ± 0.527</td>
</tr>
<tr>
<td>SW48</td>
<td>Primary</td>
<td>1.24 ± 0.053</td>
</tr>
<tr>
<td>RKO</td>
<td>Primary</td>
<td>2.49 ± 0.142</td>
</tr>
<tr>
<td>HCT116</td>
<td>Primary</td>
<td>4.45 ± 0.510</td>
</tr>
</tbody>
</table>

*IC50 values generated from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assays following exposure to dasatinib for 72 h (mean ± SD from three separate experiments).
inhibited by dasatinib at 200 nmol/L in either the HT29 or H630 cells (Fig. 2B). Cell migration was also inhibited by dasatinib treatment. There was a dose-dependent reduction in migration in cells treated with dasatinib, with 50 nmol/L dasatinib resulting in around a 46% inhibition of migration (Fig. 2C). This was reversible as following removal of the Src kinase inhibitor from the culture media, the cells were then able to migrate into the wound (Fig. 2C, right, shown for 100 nmol/L). Inhibition of BE cell migration was also inhibited by dasatinib (results not shown). Concentrations of dasatinib that resulted in inhibition of cell adhesion and migration correlated with a reduction in Src activity as measured by phosphorylation of Src on Tyr 419 (Fig. 1B).

Expression and Activity of Src in Human Colon Carcinomas

Although Src expression is elevated in a number of tumor cell types, and in colon cancer has been shown to be associated with tumor progression, there are no reports on the use of antibodies against the autophosphorylation site of Src (Tyr 419) as markers of Src activation in colon tumors in situ. Using a tumor array of 60 human colon carcinomas, we found a range of Src and phospho-Src Tyr 419 expression levels, with 84% of carcinomas having detectable levels of autophosphorylated Src and 92% having detectable Src levels (Table 2). A three-point scoring system was used to quantify the expression in each tumor, and representative examples are shown in Fig. 3 of the grades of phospho-Src Tyr 419 observed (Fig. 3A–D). Within tumors, all the cells were uniformly stained, and the scoring represents the intensity of staining rather than the percentage of cells expressing the protein. A much stronger staining pattern was seen with the phospho-Src Tyr 419 antibody than the antibody used to detect both phosphorylated and non-phosphorylated Src, making it difficult to quantify any differences in the ratio of phosphorylated to total Src levels in individual tumors. However, in some cases, very low levels of Src expression (Fig. 3I, +) was clearly associated with high levels of phosphorylated Src (Fig. 3E, +++). In well-differentiated tumors, Src was found at cell-cell boundaries (Fig. 3F, G shows higher magnification image), whereas a more diffuse cytoplasmic staining was seen in invasive tumors where there was a lack of colonic crypt-like structures (Fig. 3H). Very little Src autophosphorylation was seen in the mucinous carcinomas (Table 2). Thus, autophosphorylation of Src is readily detectable in a large percentage of colon adenocarcinomas and may provide a useful marker for inhibition of Src activity in tumors.

Dasatinib Inhibits FAK and Paxillin Phosphorylation

To further investigate the possible use of phosphospecific antibodies as biomarkers of Src activity, we looked at the ability of dasatinib to inhibit Src activity and the phosphorylation of known Src substrates in KM12C and HT29 cells. There was a dose-dependent inhibition of Src autophosphorylation, with concentrations of ≥50 nmol/L resulting in complete loss of phosphorylation in both cell lines (Fig. 4A and B). There was a similar inhibition of FAK Tyr 397 and paxillin Tyr 118 phosphorylation at equivalent concentrations of dasatinib. In contrast, much higher levels of dasatinib were required to inhibit the autophosphorylation of FAK on Tyr 972, with significant phosphorylation remaining even in 100 nmol/L treated cells (Fig. 4A), which correlates with the inability of dasatinib to inhibit FAK kinase activity (15). Dasatinib can, therefore, discriminate...
between the kinase domains of Src and FAK, resulting in differential phosphorylation of the FAK protein. No effects were seen on Src, FAK, or paxillin protein levels. Thus, these phospho-specific antibodies may provide useful tools to monitor Src activity in tumors.

As it is not always possible in the clinical setting to obtain tumor biopsy material, we wanted to assess these potential biomarkers in PBMCs, which are readily available during clinical evaluation of drugs. Initially, we looked for expression and phosphorylation of Src, paxillin, and FAK in human PBMCs isolated from healthy volunteers. Src was highly expressed and phosphorylated; however, FAK was expressed at lower levels, and phosphorylation of Tyr861 was very difficult to detect (Fig. 4B). We were unable to reproducibly detect phosphorylation of FAK on other Src-dependent phosphorylation sites (results not shown). Paxillin was also highly expressed in the human PBMCs, and its phosphorylation on Tyr118 was readily observed. Autophosphorylation of Src and phosphorylation of paxillin on Tyr118 were chosen for further analysis. Ex vivo treatment of PBMCs with dasatinib resulted in a down-regulation of both Src autophosphorylation and paxillin Tyr118 phosphorylation (Fig. 4C). Dasatinib treatment can, therefore, inhibit Src activity in both colon cancer cells and PBMCs.

**Inhibition of Src Activity in PBMCs Correlates with Inhibition of Kinase Activity in Tumors**

To correlate inhibition of Src in the blood stream with phosphorylation events in tumors, KM12C cells were grown as s.c. tumors in mice. Following treatment with dasatinib, phosphorylation of Src and paxillin in both the PBMCs and tumors was measured. Treatment with either 15 or 30 mg/kg dasatinib completely abolished Src autophosphorylation and phosphorylation of paxillin on Tyr118 in PBMCs isolated 2 h after administration of the drug (Fig. 5A). No effects on Src or paxillin protein levels were seen. Immunohistochemical analysis of the colon carcinoma xenografts showed strong staining for both phospho-Src Tyr419 and phospho-paxillin Tyr118 in both the vehicle-treated animals, which was completely abrogated in the tumors taken from the dasatinib-treated animals (Fig. 5B). Thus, inhibition of Src kinase activity in PBMCs correlates with inhibition of Src kinase activity in the tumor itself. Thus, PBMCs may be useful as a surrogate tissue to evaluate the inhibition of Src kinase activity by dasatinib in solid tumors. Furthermore, the doses of dasatinib used were clinically relevant as they yield drug exposures similar to those achievable in patients at tolerated doses.

**Table 2. Immunohistochemical analysis of human colon carcinoma tumor array**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Src</th>
<th>Phospho-Src Tyr419</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Mucinous carcinoma</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Signet ring carcinoma</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>%*</td>
<td>8</td>
<td>28</td>
</tr>
</tbody>
</table>

*NOTE: Tumors were stained with anti-Src or anti-phospho-Src Tyr419 antibodies and scored for expression: −, negative; +, low; ++, moderate; ++++, high. The number of tumors is shown for each.*

*Number of tumors expressed as a percentage of the total number of tumors in the array (n = 60).*

**Figure 2.** Inhibition of cell adhesion and migration by dasatinib correlates with inhibition of Src activity. Adhesion of HT29 or H630 cells to Matrigel (A) or poly-L-lysine (B) for 40 min in the absence or presence of dasatinib. Columns, mean of triplicate wells from a representative experiment; bars, SD. C, migration of H630 cells was determined over 18 h in the absence or presence of a range of dasatinib concentrations. Left, images taken at ×20 magnification at the beginning and end of the analysis and 4 h following removal of dasatinib (100 nmol/L). Right, quantification of migration. Columns, mean % migration seen in the untreated cells of three measurements taken from a representative experiment; bars, SD.
Discussion

Potential Use of Src Inhibitors in Solid Tumors

Although Src expression and activity is commonly observed in a number of epithelial tumors, including colon, there is conflicting evidence on the role of Src in the proliferation of solid tumors. For example, overexpression of constitutively active or kinase-defective mutants of Src did not alter colon or bladder tumor growth in vitro or in vivo (26–28), whereas other studies have reported a growth advantage upon overexpression of Src in vivo (29) and inhibition of proliferation in tumor cells in which Src expression was reduced using antisense (30). The conflicting nature of these results relying on molecular intervention has been confirmed by studies with small-molecule inhibitors of Src kinase activity. In the present study, we showed that the growth of only 2 of the 12 colon cancer cell lines was inhibited at concentrations of dasatinib that correlated with inhibition of kinase activity. Similarly, in a panel of non–small cell lung cancer and head and neck squamous cell carcinoma lines, the majority of the head and neck tumors were insensitive to concentrations of dasatinib that were required to inhibit Src activity (19). These authors went on to show that dasatinib induced a G1-S cell cycle block associated with induction of p27 in the sensitive cell lines, which has also been observed for other Src kinase inhibitors (19, 31). These effects on tumor cell proliferation are not restricted to dasatinib as the inability of other Src kinase inhibitors to prevent proliferation of tumor cells at concentrations that correlate with inhibition of Src kinase activity have also been reported (32–34). Thus, only a small subset of tumors may be dependent on Src for proliferation and, indeed, survival as both dasatinib and PP2 have been shown to induce apoptosis in certain cell lines (19, 35). The involvement of Src in growth factor–induced mitogenesis has been known for many years (4), and the interaction of Src with growth factor receptors in tumor cells (35, 36) suggests that Src may cooperate with other signaling pathways to drive proliferation of certain tumor types.

The ability of Src kinase inhibitors to block proliferation at relatively high micromolar concentrations most likely represents inhibition of other target kinases, including mitogenic growth factor receptors, at these concentrations (15). This is supported by the inhibition of cell proliferation at high nanomolar concentrations seen in the SYF−/− cells, which lack all Src family members (Fig. 1).

Although the effects of Src kinase inhibition on tumor cell proliferation are wide ranging, one consistent finding is that interference with Src activity alters the invasive and metastatic potential of tumor cells (20, 26, 33, 37, 38). We show that the effects of dasatinib on tumor cell adhesion and motility are seen at low nanomolar concentrations and are independent of effects on cell proliferation. At these concentrations of dasatinib, not only was Src kinase activity inhibited, but phosphorylation of both FAK and paxillin was also prevented. Both Src and FAK are required for cell motility, which has been linked to their ability to regulate focal adhesion turnover (25, 39–41). Upon integrin engagement, FAK becomes phosphorylated on Tyr397, creating a high-affinity binding site for Src, which then phosphorylates FAK on specific tyrosine residues. The resulting Src/FAK complex phosphorylates a number of other focal adhesion proteins, including paxillin on Tyr118, and also recruits a number of other signaling proteins to the complex. This Src-FAK signaling complex has been shown to control both cell motility and invasion (42–47), and the Src-dependent phosphorylation of FAK is required for focal

Figure 3. Expression of Src in human colon carcinomas. Immunohistochemical analysis of phospho-Src Tyr419 (A–E) and total Src (F–I) in human colon carcinomas. Magnification, × 100. Staining intensity was scored on a three-point scale. Representative images. A, negative; B and F, +; C, ++; D and E, +++; G, higher magnification image of Src at cell-cell boundaries from marked area in F.
adhesion turnover and changes in tumor cell behavior associated with an epithelial-to-mesenchymal transition (24, 28, 48). Paxillin phosphorylation is known to be Src/FAK dependent (49) and has been implicated in the motility of a number of different tumor types (50, 51). The coordinate down-regulation of Src, FAK, and paxillin phosphorylation in dasatinib-treated cells at concentrations that prevent adhesion and motility coupled with the anti-invasive activity of dasatinib reported previously, and the identification of elevated FAK and paxillin phosphorylation in highly metastatic cells (52), suggests that inhibition of these signaling pathways provides an attractive mechanism to prevent invasive and metastatic spread of tumors. In addition to effects on tumor proliferation, dasatinib may therefore have wide spread use as an anti-invasive and anti-metastatic agent.

**Development of Biomarkers for Use in Clinical Assessment of Src Kinase Inhibitors**

To aid the development of dasatinib as a treatment for patients with solid tumors, it is necessary to develop biomarkers of its activity, which can be used clinically to assess both biological efficacy and to determine the optimal therapeutic dose. Autophosphorylation of Src represents a robust read-out of Src activity (53), and we have shown that it is readily detectable in 84% of human colon adenocarcinomas studied (Table 1). Furthermore, we saw inhibition of Src Tyr419 phosphorylation in tumors taken from mice receiving dasatinib at a dose of 15 mg/kg, which produces systemic drug exposure (C\text{max} \sim 300 \text{ nmol/L and area under the curve \sim 1.5 \mu mol/L h}) similar to those observed in patients in the current phase I clinical trial in solid tumors. Our data also suggest that the phosphorylation status of FAK and paxillin are valid read-outs of dasatinib activity. There is a considerable amount of evidence linking aberrant expression of FAK with malignant disease, with elevated FAK protein reported in an increasing list of human epithelial cancers (54), which has been linked to reduced survival (55, 56). However, little is known of the phosphorylation status of FAK and paxillin in human tumors, and this is currently being evaluated.

Although we have shown that dasatinib can inhibit Src kinase activity in tumors in experimental animals, it is likely that surrogate tissues will be required for pharmacodynamic studies in patients. Peripheral blood provides a readily available and abundant source of tissue, and our analysis of Src-dependent phosphorylation events in human PBMCs showed that autophosphorylation of Src and phosphorylation of paxillin on Tyr118 provided...
The identification of robust biomarkers for dasatinib in solid tumors shown here is integral to the development of this and other therapeutics that target Src kinase. However, a number of other key issues need to be addressed, such as which patients will benefit from dasatinib treatment. Although overexpression of Src in cell lines does not predict sensitivity to Src inhibitors, identification of oncogenic pathway signatures using microarray analysis in tumors may provide a useful approach to selecting appropriate patients for therapy with targeted agents, such as dasatinib (58). Furthermore, dasatinib may also have exciting activity as an anti-invasive and anti-metastatic agent, which has yet to be addressed.

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Src Kinase Inhibition in Colon Cancer


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Molecular Cancer Therapeutics

Identification of potential biomarkers for measuring inhibition of Src kinase activity in colon cancer cells following treatment with dasatinib


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