Signaling and Regulation

Imatinib Upregulates Compensatory Integrin Signaling in a Mouse Model of Gastrointestinal Stromal Tumor and Is More Effective When Combined with Dasatinib

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Abstract
Activating mutations in the Kit receptor tyrosine kinase are associated with gastrointestinal stromal tumor (GIST). Imatinib inhibits Kit and is front-line therapy for GIST. However, imatinib most often elicits a partial response or stable disease, and most GIST patients who initially respond to imatinib eventually acquire resistance. Thus, improved treatment strategies for GIST are needed. We investigated the role of Src family kinases (SFK) in tumorigenesis in a mouse model of human GIST. The SFKs Src and Lyn were active in GIST, and surprisingly, imatinib treatment stimulated their phosphorylation/activation. We show that integrin signaling activates focal adhesion kinase and, consequently, SFKs in GIST and that imatinib enhances integrin signaling, implying a role for the extracellular matrix and integrin signaling in tumor maintenance and imatinib resistance. Dasatinib, an inhibitor of SFKs and Kit, inhibited SFK and focal adhesion kinase activation in GIST but also inhibited Kit and Kit-dependent downstream signaling pathways including phosphoinositide 3-kinase and mitogen-activated protein kinase, but not signal transducer and activator of transcription (STAT) signaling. Whereas dasatinib and imatinib alone both produced a minimal histopathologic response, combination therapy improved their efficacy, leading to increased necrosis in GIST. These results highlight the importance of SFK and STAT signaling in GIST and suggest that the clinical efficacy of imatinib may be limited by the stimulation of integrin signaling.

Introduction
Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract. GISTs express the Kit receptor tyrosine kinase (Kit) and are thought to derive from Kit+ or Kitlow interstitial cells of Cajal or their progenitors (1-3). Kit gain-of-function mutations play a critical role in GIST development and maintenance and are found predominantly in the juxtamembrane domain of the Kit receptor. However, mutations in the extracellular and kinase domains of Kit have also been described but are less frequent (4-6). We had produced a mouse model of GIST by a knock-in insertion in the mouse genome of a Kit-activating mutation, KitV558Δ, found in a case of human familial GIST syndrome (7). The KitV558Δ mutation is located in the juxtamembrane domain of Kit. Heterozygous mutant KitV558Δ/+ mice develop GIST with 100% penetrance and eventually die from complications of the disease.

Imatinib mesylate (Gleevec), an inhibitor of the Kit, platelet-derived growth factor receptor (PDGFR), and BCR-ABL tyrosine kinases, is used successfully to treat patients with GIST and chronic myelogenous leukemia (8, 9). In most GIST patients, imatinib elicits a partial response or stable disease (9). Treatment of GIST mice with imatinib produces a similar response (10). However, the molecular consequences of Kit inhibition by targeted therapy with imatinib are less well understood. Studies of Kit receptor signaling in cell lines and primary cell culture systems have identified several Kit ligand-mediated signaling cascades including phosphoinositide 3-kinase (PI3K; refs. 11-14), Src family kinases (SFK; refs. 14, 15), tyrosine phosphatases (SHP-1 and SHP-2; ref. 16), and phospholipase Cγ-1 (11) as proximal signaling initiators. Furthermore, Kit activates the mitogen-activated protein kinase (MAPK), Cbl, and signal transducer and activator of transcription (STAT) signaling pathways.

We had used imatinib to block oncogenic Kit signaling in mouse GIST to identify downstream effectors of Kit signaling in vivo. Imatinib was shown to abrogate cell cycle progression concomitant with an increase in apoptosis in tumor lesions. Furthermore, biochemical analysis of tumor tissue from imatinib-treated mice showed impairment of PI3K and mammalian target of rapamycin signaling (10).
In addition, gene expression profiles showed close similarity between mouse and human GIST and revealed roles for cell cycle regulators and IFN-inducible genes in GIST (10).

At present, imatinib therapy is first-line treatment for advanced GIST patients. Unfortunately, half of the patients treated with imatinib develop disease progression after >2 years (17). The predominant mechanism of acquired resistance to imatinib is the acquisition of second-site mutations in the Kit kinase domain (17, 18). Second-line therapy for imatinib-resistant GIST patients is sunitinib, a more broadly active receptor tyrosine kinase inhibitor, which inhibits some imatinib-resistant Kit mutants but also PDGFR, vascular endothelial growth factor receptor, RET, colony-stimulating factor-1 receptor, and flt3. However, sunitinib has been shown to delay disease progression and to extend the overall survival of GIST patients only by a median of 6 months (19). Therefore, the development of new strategies for the treatment of GIST is urgently needed.

Here, the role of SFK and integrin signaling in the mouse KitV558Δ/+ GIST model was investigated. We show that Src, Lyn, and focal adhesion kinase (FAK) are expressed and active in GIST, suggesting a role for integrin signaling in their activation, whereas imatinib treatment stimulates SFK and FAK activation and thus integrin signaling. Furthermore, the usefulness of the Kit/SFK inhibitor dasatinib in GIST was investigated. Dasatinib inhibited Kit and some Kit-dependent downstream pathways as well as integrin signaling. Dasatinib alone elicited a histologic response similar to that with imatinib, but when given in combination with imatinib, it showed improved efficacy. Therefore, combination treatment of GIST with receptor tyrosine kinase inhibitors that target different effectors of Kit signaling may improve clinical efficacy in the treatment of GIST.

Materials and Methods

Mice

Heterozygous KitV558Δ/+ mice have been described (7). The Kit+/+ mice used in these experiments have been backcrossed to C57BL/6J for 11 to 13 generations. All procedures were approved by the Institutional Animal Care and Use Committee at Memorial Sloan Kettering Cancer Center and conform to the legal mandates and federal guidelines for the care and maintenance of laboratory animals.

Drug treatment of heterozygous KitV558Δ/+ mice

Imatinib (Gleevec, STI571) was kindly provided by Novartis. Imatinib was dissolved in water as a 10 mmol/L solution. Dasatinib was synthesized as described by Shah et al. (20) and was dissolved in water as a 4 mg/mL solution. For 6-hour treatment regimens, heterozygous KitV558Δ/+ mice were treated by i.p. injection with a single dose of 45 mg/kg imatinib or a single dose of 25 mg/kg dasatinib for monotherapy; for combination treatment with both imatinib and dasatinib, mice were treated with one dose of 45 mg/kg imatinib by i.p. injection plus one dose of 25 mg/kg dasatinib by gavage orally. For longer treatments, KitV558Δ/+ mice were treated by i.p. injection with 45 mg/kg imatinib twice daily for 7 days or with 25 mg/kg dasatinib twice daily for 10 days for monotherapy; for combination treatment with both imatinib and dasatinib, imatinib was administered by i.p. injection at 45 mg/kg and dasatinib by gavage orally at 25 or 35 mg/kg twice daily for 7 days.

After the indicated treatment time periods, mice were sacrificed 6 hours after the last administration of the drug and the tumors were then quickly harvested and fixed in freshly prepared 4% paraformaldehyde for histology and immunohistochemistry or quickly frozen in liquid nitrogen for protein analyses. Seven to ten mice were used per treatment group. The histologic response to treatments was based on microscopic findings of necrosis and increased stromal fibrosis and was scored for each tumor as minimal or none (<10% response), mild (10-50% response), moderate (50-90% response), or very good (≥90% response), as described in ref. 10.

Histologic, immunohistochemical, and statistical analyses

Microscopic and immunohistochemical analyses were as described previously (10). Sections (5 μm) obtained from paraffin-embedded tissues were stained with H&E for histologic response or subjected to immunohistochemistry. The antibodies used for immunohistochemistry were cleaved caspase-3, phospho (P)-ribosomal protein S6 (S235/236), and P-MAPK [extracellular signal–regulated kinase (ERK)-1/2] (Cell Signaling; Ki67 (Novacastra); and P-FAK (Santa Cruz Biotechnology). Statistical analysis was as described previously (10). To determine cell proliferation and apoptosis in tumors, Ki67- and cleaved caspase-3–positive cells were counted under a microscope in 20 fields for at least five different tumors for each time point. The Student t test assuming unequal variances between the two samples was used to determine the significance of differences of proliferating and apoptotic cells between untreated, imatinib–treated, and dasatinib–treated GISTs. Groups were judged to differ significantly at P < 0.05. To determine the intensity of fluorescence of P-FAK, fixed frozen tumor sections from five untreated, five imatinib–treated, and three dasatinib–treated mice were subjected to immunofluorescence staining with the P-FAK Tyr576/577 antibody (Santa Cruz Biotechnology) followed by a secondary antibody conjugated to Alexa fluor 488. The fluorescence in every sample was captured by scanning the sections with Mirax Scan. Twenty-five pictures at ×20 magnification were selected from the scan for all untreated, imatinib–treated, and dasatinib–treated samples and analyzed with the MetaMorph software to determine the intensity of fluorescence in each picture. The Student t test assuming unequal variances between two samples was used to determine the significance of differences of P-FAK fluorescence between untreated, imatinib–treated, and dasatinib–treated GISTs. Groups were judged to differ significantly at P < 0.05. For real-time PCR, triplicate values from GIST of three untreated, imatinib–treated, and dasatinib–treated mice were analyzed. The Student t test assuming unequal variances between the two samples was
used to determine the significance of differences in the expression of integrins and integrin ligands between untreated, imatinib-treated, and dasatinib-treated GISTs. Groups were judged to differ significantly at $P < 0.05$ and were annotated with the symbol **. For densitometry of Western blots, specific bands were measured with Image Gauge from four to eight samples per condition. For each specific protein, the phosphorylated bands were adjusted to the total amount of the protein in each well. The data were then averaged and the untreated samples were assigned a 100% phosphorylation arbitrary value. Samples treated with imatinib, dasatinib, and imatinib plus dasatinib were compared with the untreated samples as a percentage of phosphorylation. The Student $t$ test assuming unequal variances between the two samples was used to determine the significance of differences of protein phosphorylation between untreated GISTs and GISTs treated with imatinib, dasatinib, and imatinib plus dasatinib. Groups were judged to differ significantly at $P < 0.05$ and were annotated with the symbol **.

**Immunoprecipitation and Western blotting**

Tumor lysates were prepared as described previously with the following modifications: the snap-frozen tumor was first homogenized in the PowerGen 700 homogenizer (Fisher Scientific) and then dounce homogenized 20 times and incubated on ice for 30 minutes. Lysates were cleared by centrifugation at 4°C for 30 minutes and then fractionated by SDS-PAGE for Western blotting. For immunoprecipitations, 100 μg of lysate were incubated with 4 μL of antibody in a volume of 200 μL with gentle rocking overnight at 4°C. Thirty microliters of 50% bead slurry G Sepharose (GE healthcare) were added for 2 hours at 4°C and then centrifuged for 30 seconds, and the pellet was washed five times with lysis buffer. The antibodies used for Western blot and immunoprecipitation included phospho-p44/42 MAPK (Thr202/Tyr204), p44/42 MAPK, phospho-S6 protein (Ser235/236), S6 protein, phospho-Akt (Thr308), Akt, phospho-STAT3 (Tyr705), STAT3, phospho-STAT5 (Tyr925), STAT5, phospho-Kit (Y719), Src-PY416 (P-SFK), Src, Lck, and phospho-p130 CAS (Tyr410; Cell Signaling Technologies); phospho-FAK (Y861; Millipore); p130 CAS (BD Biosciences); and phospho-FAK (Y576/577), FAK, Kit, Lyn (sc-15), and integrin β5 (Santa Cruz Biotechnology).

**Real-time PCR**

Two micrograms of total RNA were reverse-transcribed at 42°C for 30 minutes using the iScript cDNA Synthesis kit (Bio-Rad). Forty nanograms of resultant cDNA were used in a quantitative PCR reaction using an iCycler (biorad) and predesigned TaqMan Gene expression assays (assay IDs: Blk, Mm00432074_m1; Fyn, Mm00433373_m1; Src, Mm00436783_m1; Fgr, Mm00438949_m1; Hck, Mm00439302_m1; Yes1, Mm00501523_m1; Lck, Mm00802897_m1; Lyn, Mm00802933_m1; Itgb1, Mm01253227_m1; Itga2, Mm00434371_m1; Itgα6, Mm00434375_m1; Itgαv, Mm00434506_m1; Itgb5, Mm00439825_m1; Itgα3, Mm00442890_m1; Itgβ3, Mm00443980_m1; L1cam, Mm00493049_m1; Vtn, Mm00495976_m1; col1α2, Mm01165187_m1; fn1, Mm01256744_m1). Primers were chosen based on their ability to span the 3′-most exon-exon junction. Amplification was carried out for 40 cycles (95°C for 15 seconds, 60°C for 1 minute). To calculate the efficiency of the PCR reaction and to assess the sensitivity of each assay, 7-point standard curves were established (10, 3.3, 1.1, 0.37, 0.123, 0.041, and 0.015 ng). Triplicates CT values were averaged, and the amounts of the targets were interpolated from the standard curves and normalized to HPRT (hypoxanthine guanine phosphoribosyltransferase).

**Results**

**Imatinib inhibits Kit receptor signaling and activates SFKs in mouse Kit$^{V558Δ/+}$ GIST**

The Kit receptor is known to bind and activate SFKs and their respective signaling cascades in several cell systems. To investigate the role of Kit-mediated SFK signaling in mouse GIST, we performed quantitative PCR for all SFKs expressed in tumor tissue. This showed that Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, and Yes were expressed in total RNA (not shown). Next, we analyzed the activation status of the SFKs. An excellent measure for Src activation is the phosphorylation of Src tyrosine 416, Src-PY416, in the activation loop of the kinase. Using a polyclonal anti–Src-PY416 antibody, which recognizes Src-PY416 and cross-reacts widely with the activation loop phosphotyrosines of SFK members, P-SFK, we found that SFKs are expressed and activated in mouse GIST (Fig. 1A, lanes 1-4). To assess individual variability, tumor extracts were prepared from several mice and analyzed individually as previously described (10). Immunoprecipitation analysis with individual SFK antibodies and Western blotting with the anti–Src-PY416 antibody identified Src and Lyn to be expressed and activated in GIST (Fig. 1C, lanes 1 and 2). The finding that SFKs are expressed and active in GIST raised the question if their activation resulted from oncogenic Kit signaling. Because imatinib blocks oncogenic Kit signaling, we sought to determine if imatinib treatment affected SFK activation and signaling in mouse GIST. Kit$^{V558Δ/+}$ GIST mice were treated with imatinib for 6 hours, and tumor extracts analyzed as reported previously (10). Kit autophosphorylation was diminished by imatinib treatment, indicating that the drug inhibited Kit signaling; however, contrary to our expectation, imatinib treatment increased the phosphorylation of SFK activation loop tyrrosines (Fig. 1A). This was a surprising finding because we did not expect imatinib, an inhibitor of the Kit, PDGFR, and ABL kinases, to activate SFKs in GIST. A dose-response analysis showed that SFK phosphorylation increased whereas Kit tyrosine 719 autophosphorylation decreased in parallel with increasing imatinib dose, thus showing that there was a good correlation between Kit inhibition and SFK activation in GIST on imatinib treatment (Fig. 1B). Imatinib did not induce SFK activation by a
transcriptional mechanism because the gene expression profile of all SFKs in imatinib-treated GIST was unchanged compared with that in placebo-treated GIST (10). These data suggested that posttranslational modifications were responsible for SFK activation by imatinib. To identify the SFKs whose activation was increased by imatinib treatment, tumor lysates from untreated and treated animals were subjected to an immunoprecipitation analysis. Tumor lysates were immunoprecipitated with different SFK-specific antibodies and fractionated by SDS-PAGE, and then the broadly reacting anti-Src-PY416 antibody was used to identify activated SFKs by Western blot analysis. Whereas Src and Lyn were expressed and activated in GIST (Fig. 1C), Lck and Hck were expressed but not activated (data not shown). Following imatinib treatment, P-Lyn and P-Src levels were increased. Taken together, these results show that Src and Lyn are active in GIST and that imatinib treatment increases their activity.

Dasatinib inhibits both Kit receptor and SFK signaling in mouse Kit<sup>V558ΔmA</sup> GIST

The SFK inhibitor dasatinib was originally evaluated for the inhibition of imatinib-resistant BCR-ABL. Dasatinib had been shown to have broad antiproliferative effects and is known to bind to and inhibit multiple kinases including SRC, ABL, and Kit (21). Therefore, dasatinib might be effective in inhibiting imatinib-resistant Kit mutants as well as downstream effectors of oncogenic Kit signaling. Particularly, dasatinib might inhibit the consequences of imatinib-induced SFK activation and thus show improved efficacy in GIST compared with imatinib. To investigate the effect of dasatinib on oncogenic signaling in GIST, Kit<sup>V558ΔmA</sup> mice were treated by i.p. injection of 25 mg/kg dasatinib twice daily and treatment outcome was compared with that in mice treated with 45 mg/kg imatinib as previously described (10). After 10 days of treatment, dasatinib induced arrest of cell proliferation, similar to imatinib (Fig. 6A). However, in contrast to imatinib, dasatinib did not induce apoptosis in GIST (Fig. 6B). H&E staining of dasatinib-treated tumors showed a minimal to mild pathologic response, similar to imatinib-treated GIST mice on the C57BL/6J background (Table 1). To investigate the signaling pathways that are affected by dasatinib after a short treatment period, GIST mice were treated for 6 hours and tumor extracts were prepared from several mice and analyzed individually as previously described (10). Because the Kit<sup>V558ΔmA</sup> mice carry a gain-of-function Kit allele responsible for tumor development, we first analyzed Kit receptor activation by monitoring the autophosphorylation of Kit-Y719 by Western blotting. A reduction of Kit-Y719 phosphorylation was observed in the dasatinib-treated samples compared with untreated controls, indicating that dasatinib inhibits Kit receptor activation in Kit<sup>V558ΔmA</sup> mice (Fig. 2A). We then evaluated inactivation/activation of...

![Image](image.png)
SFKs, and SFK docking is a mechanism of Kit-mediated autophosphorylation site Y567 is a known docking site for and that their activation is increased by imatinib. The Kit anti–Src-PY416 antibody showed that dasatinib treatment similarly inhibited the serine threonine kinase Akt, and ribosomal protein S6 phosphorylation was found to be reduced as well (Figs. 2A and 3). ERK1/ERK2 phosphorylation was not reduced (Figs. 2A-B and 7B); only the phosphorylation was reduced by dasatinib. However, in contrast to imatinib, Src and Lyn phosphorylation in dasatinib-treated GIST mice, STAT5 and STAT3a phosphorylation was also reduced by dasatinib. However, in contrast to imatinib, in dasatinib-treated GIST mice, STAT5 and STAT3a phosphorylation was not reduced (Figs. 2A-B and 7B); only the phosphorylation of the STAT3b isoform was slightly reduced. Next, we investigated the effect of dasatinib on SFKs and their state of activation in GIST. Using the polyclonal anti–Src-PY416 antibody, we found that dasatinib treatment abolished activation of SFKs in GIST (Fig. 2C). Immunoprecipitation analysis with the individual SFK antibodies and subsequent Western blotting with the anti–Src-PY416 antibody showed that Src and Lyn phosphorylation was inhibited by dasatinib treatment (Figs. 1C and 2D). These results indicate that dasatinib affected the PI3K and MAPK signaling cascades as well as SFK signaling in GIST, but not STAT signaling.

**Imatinib activates integrin signaling**

Our results indicate that Src and Lyn are active in GIST and that their activation is increased by imatinib. The Kit autophosphorylation site Y567 is a known docking site for SFKs, and SFK docking is a mechanism of Kit-mediated SFK signaling (14). Thus, Kit could directly activate SFKs in GIST. However, our dose-response analysis showed that SFK activation correlated with Kit inhibition, making direct activation of SFKs by Kit unlikely. To determine if Kit associates with SFKs in GIST and has a role in SFK activation, tumor lysates from untreated, imatinib-treated, and dasatinib-treated GIST mice were subjected to a coimmunoprecipitation analysis. Western blots of fractionated Kit immunoprecipitates analyzed with the anti–Src-PY416 antibody did not reveal any bands, indicating that Kit antibody did not precipitate any activated SFKs (Fig. 4A) and thus suggesting that activation of SFKs in GIST is not directly mediated by the Kit receptor.

An alternative way to activate SFKs could be through integrin signaling. FAK is a critical mediator of integrin signaling in part through phosphorylation by Src. FAK-Src signaling may mediate cell survival, proliferation, and motility in normal cells, and it was found to be activated in many tumor cells and to generate signals for tumor growth and metastasis (22–24). Therefore, it is possible that in GIST, SFKs are activated via integrin signaling. To investigate this possibility, we determined the phosphorylation status of FAK. FAK Y397 is phosphorylated on activation of FAK by integrin clustering. Subsequently, SFKs bind to FAK Y397, become activated, and phosphorylate other tyrosine residues of FAK. FAK was found to be phosphorylated, and imatinib treatment induced a slight but consistent increase in Y397 phosphorylation. In addition, imatinib treatment leads to an increase in the phosphorylation of FAK at Y576/577 in the activation loop and at Y861 (Fig. 4B). This indicates that FAK is active in GIST and that imatinib stimulates FAK signaling. In agreement with this, the downstream effector of FAK-Src signaling, the adaptor protein p130 CAS, was phosphorylated in GIST at Y410 and phosphorylation was increased by imatinib treatment (Fig. 4B). Dasatinib treatment also induced an increase in Y397 phosphorylation, but in

### Table 1. Individual histologic response of GIST lesions in KitV558Δ/+ mice to treatment with imatinib or dasatinib alone or imatinib/dasatinib combinations

<table>
<thead>
<tr>
<th>Imatinib</th>
<th>Dasatinib</th>
<th>Imatinib + Dasatinib (25 mg/kg)</th>
<th>Imatinib + Dasatinib (35 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild (#469)</td>
<td>Mild (#317)</td>
<td>Mild to moderate (#537)</td>
<td>Moderate (#571)</td>
</tr>
<tr>
<td>Very minimal (#350)</td>
<td>Mild (#354)</td>
<td>Mild (#538)</td>
<td>Moderate (#573)</td>
</tr>
<tr>
<td>None (#339)</td>
<td>Very minimal (#358)</td>
<td>Moderate (#529)</td>
<td>Moderate (#575)</td>
</tr>
<tr>
<td>None (#336)</td>
<td>Focal mild (#362)</td>
<td>Moderate with focal necrosis (#540)</td>
<td>Moderate with focal necrosis (#572)</td>
</tr>
<tr>
<td>Mild (#239)</td>
<td>Focal mild (#365)</td>
<td>Moderate (#541)</td>
<td>Moderate (#585)</td>
</tr>
<tr>
<td>None (#465)</td>
<td>None (#368)</td>
<td>Mild (#542)</td>
<td>Moderate (#586)</td>
</tr>
<tr>
<td>None (#471)</td>
<td>None (#371)</td>
<td>Mild to moderate (#539)</td>
<td>Moderate with focal necrosis (#574)</td>
</tr>
</tbody>
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**NOTE:** Seven mice in each group were treated for 7 d with imatinib (45 mg/kg), 10 d with dasatinib (25 mg/kg), or 7 d with combinations of imatinib (45 mg/kg) and dasatinib at two different doses of dasatinib (25 or 35 mg/kg). The histologic response to the treatments was assessed based on microscopic findings of necrosis and increased stromal fibrosis and was scored for each tumor as minimal or none (<10% response), mild (10-50% response), moderate (50-90% response), or very good (≥90% response), as described in ref. 10. Individual tumor samples are identified by the symbol # and the observed histologic response is indicated.
contrast to imatinib, phosphorylation of FAK Y576/577 and Y861 was inhibited by dasatinib, in keeping with the dasatinib activity as an SFK and a FAK inhibitor (Fig. 4B; ref. 25). In addition, immunohistochemistry of GIST sections with anti-phospho-FAK antibody confirmed the activation of FAK by imatinib and its inhibition by dasatinib in the tumor (Fig. 3G-I). The tumor cells were found to be strongly positive for staining with anti-Kit antibody (Fig. 3J-L). This indicated that imatinib stimulated FAK activation in Kit-expressing tumor cells rather than in cells of the tumor microenvironment, which are Kit negative.

To determine whether FAK is in a complex with SFKs in GIST, a coimmunoprecipitation analysis using anti-FAK antibody was done next. Fractionation of FAK immunoprecipitates and Western blotting with SFK antibodies identified Src and Lyn in tumor extract. Western blot analysis with anti–Src-PY416 antibody indicated that the Src and/or Lyn proteins, which are associated with FAK, were active in untreated mice. Furthermore, in the imatinib-treated samples, phosphorylation of P-SFK appeared to be increased, whereas no phosphorylation was observed on treatment with dasatinib (Fig. 4C). This indicated that FAK-Src and/or FAK-Lyn complexes were stimulated by imatinib and inhibited by dasatinib.

Integrins are heterodimeric transmembrane receptors composed of an α and a β subunit. We assessed the expression of different integrin subunits in GIST using quantitative PCR analysis to determine if the level of integrin expression was affected by imatinib or dasatinib treatment (Fig. 5A). This analysis showed high expression of integrins αv and β1 in untreated GIST, although integrins α3, β3, and β5 were present as well. Imatinib and dasatinib treatment did not change the level of expression of integrins αv and β1, but increased the level of expression of integrins α3, β3, ζ3, and α6. We further examined the levels of expression of different integrin ligands. Integrin ligands
known to activate FAK such as collagen, fibronectin, and vitronectin were expressed in GIST, but their levels of expression were not altered by drug treatment. Expression of L1cam, a ligand for integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$, has been reported in GIST patients (26). Expression of L1cam was found in untreated GIST, and surprisingly, the level of expression of L1cam was dramatically increased after imatinib and dasatinib treatment (Fig. 5B). These results imply that imatinib and dasatinib treatment activates integrin signaling by elevating the mRNA expression of some integrins and/or the integrin ligand L1cam. Taken together, these results indicate that integrins activate FAK in GIST and that imatinib and dasatinib treatment stimulates the first step in FAK activation, the phosphorylation of Y397. FAK-Lyn and FAK-Src complexes are formed and active in GIST, but imatinib further activates these dual kinase complexes. FAK-SFK complexes are also formed in dasatinib-treated mice; however, they are not active due to the inhibitory activity of dasatinib against SFK (25).

**Treatment of KitV558Δ/+** GIST mice with imatinib plus dasatinib produces an increased clinical response

Given the differing biochemical consequences of imatinib and dasatinib treatment in GIST, we investigated whether treatment with both drugs together would be beneficial. GIST mice were treated simultaneously for 7 days with imatinib (45 mg/kg) and dasatinib (25 mg/kg) twice daily, both doses representing close to a maximal tolerated dose in mice. Immunohistochemical analysis of tumor tissue indicated a complete arrest of tumor cell proliferation
determined by Ki67 staining, consistent with the effect seen with each drug individually (Fig. 6A). However, a net increase in apoptosis was observed in comparison with imatinib monotherapy (Fig. 6B). Furthermore, H&E staining of tumor sections showed a consistently improved histologic response compared with treatment with imatinib or dasatinib alone, assessed as a moderate response with focal areas of necrosis (Fig. 6C; Table 1). The histologic response of combination treatment was even stronger if the dasatinib dose was increased to 35 mg/kg. These results indicate that combination treatment of imatinib plus dasatinib is beneficial compared with imatinib monotherapy in GIST mice.

Finally, we investigated the consequences of combination therapy on oncogenic signaling pathways in GIST. As expected, phosphorylation of Kit, Akt, ribosomal protein S6, and ERK1/2 was reduced by the combination treatment, as is the case for each drug individually. Phosphorylation of SFK tyrosine 416 was also reduced by the double treatment, indicating that dasatinib can overcome the activation of SFKs seen with imatinib treatment (Fig. 7A and B). Furthermore, phosphorylation of the STAT3a, STAT3b, and STAT5 transcription factors was diminished as well. Taken together, these results indicate that simultaneous treatment with imatinib and dasatinib improved treatment outcome in an additive manner and downregulated four oncogenic cascades in GIST: the PI3K, SFK, MAPK, and STAT pathways. Furthermore, these results imply that the simultaneous downregulation of several pathways is required to achieve a good histologic/clinical response.

**Discussion**

Mice carrying a KitV558A germ-line mutation in the juxtamembrane domain of Kit had been generated based on a
case of human familial GIST with the same mutation. Because juxtamembrane domain mutations in Kit represent a majority of somatic Kit mutations in GIST, the KitV558Δ/+ mouse is an excellent tool to assess new treatment strategies to enhance imatinib efficacy. By using the KitV558Δ/+ GIST mouse, we have previously shown that imatinib inhibits cell cycle progression and induces apoptosis, as well as inhibits the translational response, MAPK signaling, as well as STAT3 and STAT5 activation (10). As Kit is known to activate SFK signaling, we investigated the effect of imatinib treatment on SFK signaling in mouse GIST. Interestingly and surprisingly, we found that imatinib, an inhibitor of the Kit, PDGFR, and ABL kinases, induces increased activation of SFKs, in particular Lyn and Src, as well as FAK in mouse GIST. We have investigated the mechanisms of SFK activation in GIST and its stimulation on imatinib treatment. SFKs are known to associate with and act as effectors of different cell membrane receptors including receptor tyrosine kinases, cytokine receptors, and integrins. Because Kit-PY567 is a docking site for SFKs, it was possible that Kit may activate SFKs even in the presence of imatinib. However, this is unlikely because imatinib binds Kit in its inactive conformation and inhibits Kit autophosphorylation and kinase activity. In fact, in Kit immunoprecipitates of tumor lysates both from untreated and treated mice, no active SFKs could be identified.

In the tumor microenvironment, integrins and their ligands are thought to provide signals for tumor cell survival and proliferation as well as have roles in tumor progression and metastasis (27, 28). Integrin signaling is bidirectional: whereas inside-out signaling mediated by growth factors such as vascular endothelial growth factor, insulin-like growth factor, and Kit ligand may increase the affinity of integrins to extracellular ligands in the extracellular matrix, outside-in signaling mediates the cellular responses to cell adhesion. In mouse GIST, several integrin subunits are highly expressed and are thought to have roles in tumor progression and metastasis. It is likely that integrin signaling in GIST is also bidirectional and that both the integrin ligands and the Kit ligand are agonists in this signaling network, and the question arises how integrin signaling is affected by the imatinib inhibition of Kit in the mouse GIST model. Different integrin ligands including fibronectin, laminin, Vcam, vitronectin, and L1cam are expressed in mouse GIST. Interestingly, L1cam expression was upregulated by both imatinib and dasatinib treatment in our gene expression profile and quantitative PCR analysis in contrast to the other integrin ligands. Although we do not know the molecular mechanisms of increased L1cam expression by imatinib treatment because L1cam is a ligand for integrins αVβ1, α6β1, and αVβ3, which are expressed in GIST, this could explain the upregulation of integrin signaling by imatinib (29, 30). Furthermore, upregulation of expression of different integrin subunits (i.e., integrins α3, β1, α2, and αc) by imatinib and dasatinib could result in increased integrin signaling.

The cytoplasmic tyrosine kinase FAK associates with integrins in focal adhesions and is critical in signaling mediated by integrin clustering. On integrin clustering, FAK is autophosphorylated on FAK Y397, creating an SH2 binding site for SFKs and consequently inducing SFK activation. Maximal FAK activity is attained by Src-mediated phosphorylation of FAK tyrosines Y576/Y577 in the activation loop of the FAK kinase (22, 23, 31). In turn, maximal Src kinase activity is attained on phosphorylation of Src Y416 in the activation loop of the Src kinase by FAK (32). Whereas in untreated GIST, FAK Y397 is phosphorylated, imatinib treatment increased FAK Y397 phosphorylation, indicating increased integrin clustering and signaling. Furthermore, FAK Y576/Y577 was phosphorylated in untreated GIST and phosphorylation was increased on imatinib treatment, implying a role for FAK signaling in SFK activation in GIST. In addition, a protein complex was identified that includes FAK-Src and Lyn. Both the Src and Lyn tyrosine kinases are known to interact with FAK on integrin stimulation (31, 33). This complex was active because SFK Y416 was phosphorylated. Therefore,
integrin-mediated compensatory FAK-SFK signaling activated by imatinib is independent of oncogenic Kit signaling. In agreement with this, tyrosine phosphorylation of SFK, FAK, and paxilin was found to be resistant to Kit inhibition in vitro in GIST cell lines (34).

Our results imply that integrin-FAK–mediated signaling has a role in tumor maintenance and is a possible negative regulator in imatinib treatment. Interestingly, FAK is highly expressed in patients with malignant GIST (35). In a large fraction of breast cancers, the FAK gene is amplified, and mechanistic studies showed that FAK sustains tumorigenesis by mediating SFK-induced phosphorylation of p130 CAS (36). Furthermore, a ligand of integrin αV-β3, L1cam (CD171), is highly expressed in GIST, and high L1cam expression may correlate with reduced survival (26). Therefore, targeting of FAK and SFKs or their downstream effectors should be beneficial in GIST treatment.

We have used the SFK/FAK and Kit inhibitor dasatinib as a single agent and in combination with imatinib to investigate its efficacy in mouse GIST. As a single agent, dasatinib inhibits cell proliferation, Kit autophosphorylation/activation, and Kit downstream targets (the PI3K and MAPK signaling cascades) in mouse GIST. This is consistent with studies in mast cells and hematopoietic cell lines showing inhibition by dasatinib of the wild-type and juxtamembrane mutant isoforms of Kit and the imatinib-resistant activation loop mutant Kit isoforms (37, 38). In a study of kinase inhibitor selectivity, dasatinib was also found to have a better affinity for Kit wild-type and Kit V559D than imatinib (39). However, the activity of dasatinib in GIST differs from that of imatinib as follows: First, although dasatinib inhibits cell cycle progression, it does not induce apoptosis; second, whereas imatinib inhibits STAT3 and STAT5 activation, dasatinib does not; and

**FIGURE 6.** Combination treatment with imatinib plus dasatinib has additive effects in KitV558Δ/+ mouse GIST. Quantitation of proliferating (A) and apoptotic (B) cells in tumor sections of KitV558Δ/+ mice treated with placebo (ctr), 7-d imatinib (45 mg/kg), 10-d dasatinib i.p. (25 mg/kg), and 7-d imatinib (45 mg/kg) plus dasatinib orally (25 mg/kg; DAS + IM). Groups were judged to differ significantly at P < 0.05 (see Materials and Methods). C, H&E staining of GIST sections from untreated mice and mice treated with 7-d imatinib (45 mg/kg), 10-d dasatinib i.p. (25 mg/kg), and 7-d imatinib (45 mg/kg) plus dasatinib orally (25 or 35 mg/kg). Arrows, areas of moderate response with a decrease in cellularity and an increase in stroma; arrowheads, large areas of necrosis. The numbers at the bottom left correspond to the tumor samples (see Table 1).
third, in contrast to imatinib, which activates SFKs, dasatinib inhibits SFKs. These results are consistent with the high affinity of dasatinib for SFKs (25, 39).

In a recent study, dasatinib was shown to inhibit the active conformation of FAK (25). In agreement with this, dasatinib was shown to inhibit the phosphorylation of FAK activation loop tyrosines Y576/Y577, but not the phosphorylation of FAK tyrosine 397. The failure of dasatinib to inhibit Y397 phosphorylation implies that the phosphorylation of this tyrosine residue does not require a fully activated FAK kinase, or alternatively, inhibition of Src/Lyn blocks the complete activation of FAK. Therefore, these results provide a rationale for the use of dasatinib in combination with imatinib in the treatment of GIST.

The question then arises why the combination of imatinib and dasatinib produced a better histologic response than did dasatinib alone, since dasatinib inhibits Kit, SFKs, and FAK. One possibility is that STAT signaling has an important but not sufficient role in GIST maintenance. We previously showed that imatinib inhibits STAT signaling in GIST. Whereas dasatinib does not inhibit STAT3 and STAT5 phosphorylation, combination treatment diminishes the phosphorylation of STAT3 and STAT5. This may indicate that inhibition of STAT signaling results in an enhanced histologic response. In agreement with a critical role for STAT3 signaling in GIST, in colitis-associated cancer, STAT3 was shown to have a critical role in mediating cell proliferation and cell survival (40). The observation of a failure of dasatinib to inhibit STAT3/5 signaling is of interest, but an investigation of the role of STAT3/5 signaling in mouse GIST will be a challenging next step. For combination therapy, both imatinib and dasatinib were used at close to maximally tolerated doses to optimally inhibit the drug-sensitive signaling cascades. The downside of this strategy was that only additive effects of the combination therapy were identified. Clearly, careful dose-response studies will need to be done in the future to further understand the benefits of combination imatinib/dasatinib combination therapy.

Previously, we showed that imatinib inhibits PI3K, MAPK, and STAT signaling in our mouse GIST model. Here, we show that imatinib also activates SFKs by compensatory integrin signaling; furthermore, we show that dasatinib treatment inhibits Kit activation and SFK, FAK, PI3K, and MAPK signaling, but not STAT signaling. Therefore, the increased clinical response by combination treatment with imatinib and dasatinib seems to result from

![FIGURE 7](image-url). Additive biochemical effects in tumors treated with imatinib and dasatinib. A, Western blot analysis of tumor extracts from untreated mice and mice cotreated with 6 h imatinib (45 mg/kg) plus dasatinib (25 mg/kg). B, densitometric tracing analysis of Western blots of tumor extracts from mice treated with 6-h imatinib (45 mg/kg), dasatinib (25 mg/kg), and imatinib plus dasatinib. The values are percentages of phosphorylation, with untreated samples assigned an arbitrary value of 100% phosphorylation.
the simultaneous inhibition of PI3K, MAPK, STAT, and SFK signaling. It is known that tyrosine kinase inhibitors can be active against "off-targets" and we cannot exclude that such off-target effects may increase or decrease their therapeutic potential in GIST (25). However, it has been shown that a combination of two tyrosine kinase inhibitors that target BCR-ABL can preempt in vitro resistance (41). Similarly, a combination of different tyrosine kinase inhibitors could preempt imatinib resistance in GIST. Therefore, these results provide a rationale for the development of combined targeted therapies for the treatment of human GIST.

Disclosure of Potential Conflicts of Interest

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References


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