Inhibition of Src Family Kinases with Dasatinib Blocks Migration and Invasion of Human Melanoma Cells

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Abstract

Src family kinases (SFK) are involved in regulating a multitude of biological processes, including cell adhesion, migration, proliferation, and survival, depending on the cellular context. Therefore, although SFKs are currently being investigated as potential targets for treatment strategies in various cancers, the biological responses to inhibition of SFK signaling in any given tumor type are not predictable. Dasatinib (BMS-354825) is a dual Src/Abl kinase inhibitor with potent antiproliferative activity against hematologic malignancies harboring activated BCR-ABL. In this study, we show that dasatinib blocks migration and invasion of human melanoma cells without affecting proliferation and survival. Moreover, dasatinib completely inhibits SFK kinase activity at low nanomolar concentrations in all eight human melanoma cell lines investigated. In addition, two known downstream targets of SFKs, focal adhesion kinase and Crk-associated substrate (p130^{CAS}), are inhibited with similar concentrations and kinetics. Consistent with inhibition of these signaling pathways and invasion, dasatinib down-regulates expression of matrix metalloproteinase-9. We also provide evidence that dasatinib directly inhibits kinase activity of the EphA2 receptor tyrosine kinase, which is overexpressed and/or overactive in many solid tumors, including melanoma. Thus, SFKs and downstream signaling are implicated as having key roles in migration and invasion of melanoma cells. (Mol Cancer Res 2008;6(11):1766–74)

Introduction

Increased protein levels and kinase activities of Src family kinases (SFK) have been observed in a wide diversity of human cancers, including melanoma, breast, ovarian, and lung cancer (1-7). The prototype SFK is c-Src, which is a protein tyrosine kinase from which the oncogenic viral Src (*v-Src*) is derived (8). An abundance of evidence suggests that a primary role for SFKs, in particular c-Src, is to regulate cell adhesion, motility, and invasion (9-13). During tumor cell transendothelial migration, a critical step in cancer metastasis, Src becomes activated at the heterotypic contact between the transmigrating melanoma cell and the neighboring endothelial cells (14). SFKs can also promote proliferation and survival in response to signaling initiated by binding of mitogenic growth factors to their cognate receptors (15-17). In addition, there is growing evidence that SFKs have a critical role in tumor angiogenesis at least in part through regulation of expression of angiogenic factors such as interleukin-8 and vascular endothelial growth factor (18).

Dasatinib (BMS-354825) is a novel, oral, multitargeted, kinase inhibitor of BCR-ABL, c-KIT, PDGFR, and SFKs (19-21). The antitumor potency of dasatinib has been shown in early- and late-phase clinical trials for chronic myelogeneous leukemia (22, 23). Dasatinib recently has been approved by the Food and Drug Administration and the European Union for treatment of all stages of chronic myelogeneous leukemia in patients with imatinib-resistant or imatinib-intolerant disease. Clinical trials are currently ongoing for evaluation of dasatinib in treatment of solid tumors. Because of the myriad of critical roles of SFKs in basic biological processes, molecularly targeted small-molecule inhibitors of SFKs could induce numerous biological responses. Importantly, the therapeutic potential of dasatinib in solid tumors, including melanoma, remains to be fully determined. However, the development of Src transgenic mice and the effects of activated Src on tumor formation and promotion in these animal models, including that of the skin, further suggest a critical role of SFKs in solid tumors, including melanoma (24, 25). Recently, clinical trial data were presented that indicate the potential utility of dasatinib in treatment of solid tumors such as metastatic hormone-refractory prostate cancer (26).

We investigated the effect of dasatinib on eight human melanoma cell lines, all of which harbor constitutive SFK activity as measured by tyrosyl phosphorylation of their autophosphorylation site. Here, we show that treatment of melanoma cells with low nanomolar concentrations of dasatinib completely abolishes the SFK autophosphorylation activity in cells. Moreover, blockade of SFK activity correlates with greatly reduced phosphorylation of the known SFK downstream targets, focal adhesion kinase (FAK), and Crk-associated substrate (p130^{CAS}; refs. 27-29). Elevated FAK activity in human melanoma was shown previously to promote tumor cell invasion and migration (30-32). Consistent with this role of

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FAK activity in melanoma, dasatinib-mediated inhibition of the SFK/FAK signaling pathway completely abolishes migration and invasion of melanoma cells. Our findings suggest that SFK signaling is important for migration and invasion but not proliferation and survival of melanoma cells.

Results

Dasatinib Inhibits Migration of Human Melanoma Cells

Approximately 200,000 1205-Lu (Fig. 1A) or 100,000 A2058 (Fig. 1B) human melanoma cells were seeded in 12-well cell culture plates. The next day, when cultures were fully confluent, a "scratch" was made with a small pipette tip (10 μL) across the wells. The cells were then washed twice to remove any floating cells and treated with control vehicle alone (DMSO) or increasing amounts of dasatinib as indicated. Twenty hours later, photomicrographs of the scratch were taken and migration was quantified by counting the cells that migrated into the scratch area. Each number represents the average count of cells in three scratch assays (one scratch per well, one well per experiment, three independent experiments). With both 1205-Lu and A2058 cell lines, markedly fewer cells migrated into the wound in the presence of higher concentrations of dasatinib compared with the DMSO control. The inhibitory effect of dasatinib was dose dependent with an IC_{50} of ≈50 nmol/L.

Dasatinib Suppresses Invasion of Human Melanoma Cells

Invasion assays were established and optimized for A2058 (Fig. 2A) and 1205-Lu (Fig. 2B) human melanoma cell lines. Approximately 20,000 A2058 or 50,000 1205-Lu cells were seeded in 0.2% serum-containing medium on top of the Matrigel in 24-well format Boyden-Chamber invasion chambers. To promote invasion, the lower part of the chamber was filled with 100% conditioned medium containing 10% serum. Dasatinib or DMSO vehicle control was immediately added to both the upper and lower parts of the invasion chambers. Cells were allowed to invade and migrate for 24 hours. Cells that migrated to the opposite site of the invasion chamber membrane were fixed and stained. Cells in at least three different areas of the membrane were counted and the experiment was repeated two more times. The number of invading cells was lower after 24 hours in the presence of increasing amounts of dasatinib versus DMSO control (Fig. 2C). Suppression of invasion by dasatinib was dose dependent, with an IC_{50} of ≈50 nmol/L.

Dasatinib Down-Regulates Expression of Matrix Metalloproteinase-9

Three hundred thousand A2058 human melanoma cells were plated overnight in 10-cm round cell culture plates. The cells were then treated with DMSO vehicle control or increasing amounts of dasatinib dissolved in RPMI 1640 supplemented with 1% serum. Ninety-six hours after the start of treatment, supernatants were collected and MMP-9 protein expression levels were measured using the MMP-9 Biotrak activity assay system (Amersham Biosciences). As shown in Fig. 2D, dasatinib down-regulates MMP-9 protein expression in A2058 cells in a dose-dependent manner with an IC_{50} between 3 and 10 nmol/L. In addition to down-regulating total MMP-9 protein, dasatinib also blocked MMP-9 enzymatic activity at concentrations similar to the data shown in D (data not shown). Expression levels of MMP-9 were either not detectable (MeWo, Sk-Mel-5, G-361) or too low (A375, 1205-Lu, 451-Lu, Sk-Mel-28) to observe effects of dasatinib in the other melanoma cell lines (data not shown).

Dasatinib Does Not Markedly Inhibit Proliferation and Viability of Human Melanoma Cells

One thousand human melanoma cells were seeded in each well of 96-well plates overnight and treated with DMSO vehicle control or increasing amounts of dasatinib as indicated (Fig. 3). For viability assays (Fig. 3A), cells were directly incubated with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) substrate 72 hours posttreatment. For proliferation assays (Fig. 3B), cells were lysed 96 hours posttreatment and the supernatant was incubated with lactate dehydrogenase detection reagent. For both assays, absorbance was measured at 490 nm and percent viability or cell number (proliferation) was normalized to the absorbance of DMSO-treated cells. Results show that human melanoma cells are not significantly ($P > 0.05$) growth inhibited by dasatinib, even at concentrations as high as 2 μmol/L.

As a positive control for inhibition of growth and survival of human melanoma cells, we used the tyrosine kinase inhibitor PD180970. As previously reported, PD180970 had dramatic effects on both growth and survival of all human melanoma cells, even at low nanomolar concentrations (data not shown; ref. 33). Because both compounds, PD180970 and dasatinib, inhibit SFK catalytic activity at low nanomolar concentrations, we conclude that inhibition of SFK catalytic activity in melanoma cells is not sufficient to markedly influence growth and survival. Therefore, the effects of the tyrosine kinase inhibitor, PD180970, on human melanoma cell survival cannot solely be attributed to Src inhibition. Significantly, these results indicate that the effects of dasatinib seen on migration and invasion (Figs. 1 and 2) are not due to inhibition of growth and/or survival.

Dasatinib Selectively Blocks SFK Downstream Signaling in Human Melanoma Cells

To identify possible targets of dasatinib that are known to participate in migration and invasion of human melanoma cells, we first treated A2058 human melanoma cells with either DMSO vehicle control or dasatinib in a dose-dependent (Fig. 4A) and time-dependent (Fig. 4B) manner. We then performed Western blot analysis on SFK and downstream substrates of SFKs, including FAK and Crk-associated substrate, $p130^{CAS}$. Antibodies to the autophosphorylation site in c-Src (Tyr^{419} in human and Tyr^{416} in chicken) cross-react with the corresponding autophosphorylation sites in other SFKs. Tyrosyl phosphorylation of FAK and $p130^{CAS}$ is important for cell migration and invasion (27-29). The data presented here show that in addition to blocking SFK autophosphorylation, dasatinib also blocks tyrosyl

phosphorylation of the SFK downstream substrates FAK and $p130^{CAS}$. Furthermore, SFKs, FAK, and $p130^{CAS}$ are all inhibited rapidly (within 15 min) and at similar concentrations of dasatinib (IC_{50} <50 nmol/L), suggesting that SFKs signal through FAK and $p130^{CAS}$.

Because 300 nmol/L of dasatinib was sufficient to completely abolish tyrosyl phosphorylation of all three signaling proteins, we then treated eight human melanoma cell lines with 300 nmol/L dasatinib (or DMSO) for 24 h. Significantly, tyrosyl phosphorylation of SFK, FAK, and

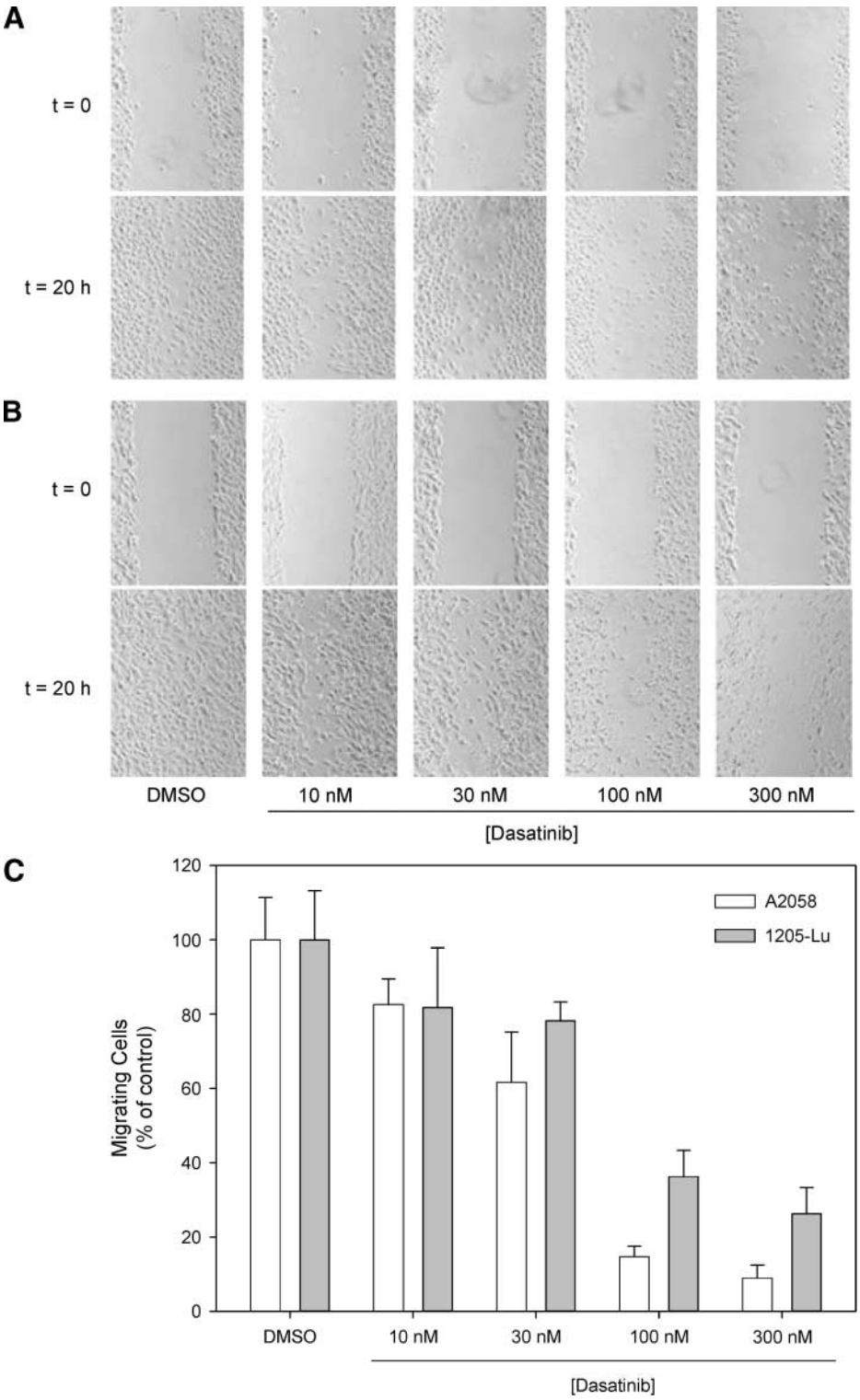

FIGURE 1. Dasatinib inhibits migration of human melanoma cells. 1205-Lu (**A**) and A2058 cells (**B**) were plated in 12-well cell culture plates and grown to 100% confluence. A single scratch was made in the confluent monolayer, floating cells were washed off, and attached cells were treated with DMSO vehicle control or increasing concentrations of dasatinib as indicated. Each scratch was photographed at $t = 0$ and again at $t = 20$ h. Cells that migrated into the scratch were counted. The data in **C** represent the average and SD of three independent experiments. In all cases, when treated samples are compared with corresponding controls, $P < 0.05$.

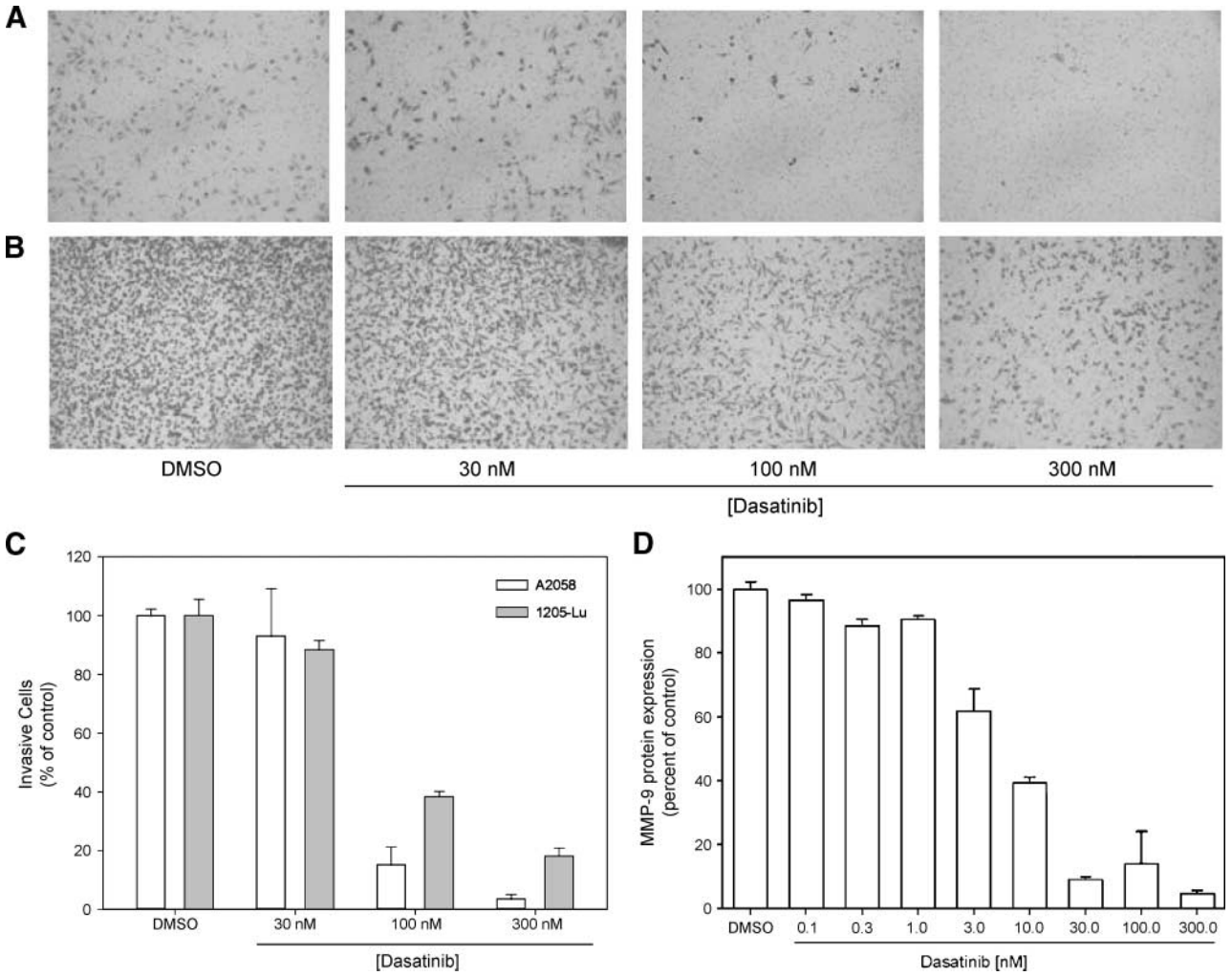

FIGURE 2. Dasatinib blocks invasion of human melanoma cells. A2058 (**A**) and 1205-Lu cells (**B**) were seeded in 0.2% serum-containing medium into the upper part of the Matrigel invasion chambers. The lower chambers were filled with 100% conditioned medium containing 10% serum to promote invasion through the Matrigel. Cells were treated immediately with DMSO vehicle control or increasing concentrations of dasatinib as indicated. Twenty-four hours later, noninvading cells were removed and the invaded cells were fixed and stained. The experiment was repeated two more times, with similar results. Cells in at least three different areas of the membrane were counted (**C**); columns, average of three independent experiments; bars, SD. To measure the effect of dasatinib on MMP-9 protein expression, A2058 cells were treated for 96 h with increasing concentration of dasatinib or DMSO control and supernatants were analyzed for total MMP-9 protein concentrations (**D**). In all cases, when treated samples are compared with corresponding controls, $P < 0.05$.

p130^{CAS} was completely inhibited in seven of eight cell lines that were treated with dasatinib (Fig. 5). In the noninvasive cell line Sk-Mel-5, tyrosyl phosphorylation of FAK and p130^{CAS} could not be detected and SFKs had the least amount of tyrosyl phosphorylation of all melanoma cells investigated, further supporting the hypothesis that FAK/p130^{CAS} signaling is involved in invasion of melanoma cells. Interestingly, known growth and survival pathways of melanoma cells, including the p44/42 mitogen-activated protein kinases Erk1 and Erk2, AKT, p38, and Stat3 signaling were not consistently inhibited by dasatinib. These results are in agreement with our findings that dasatinib does not significantly inhibit growth and survival of melanoma cells (Fig. 3). Altogether, these data show that the effects of dasatinib are generally consistent across diverse human melanoma cells and include inhibition of signaling pathways that are involved in cell adhesion, migration, and invasion.

Dasatinib Inhibits EphA2 Tyrosine Phosphorylation in Human Melanoma Cells and Blocks EphA2 Kinase Activity In vitro

EphA2 is a member of the Eph family of receptor tyrosine kinases and is overexpressed and/or overly active in several human cancers, including melanoma (34-40). Because EphA2 is reportedly involved in migration and invasion of tumor cells, we also investigated the effect of dasatinib on EphA2 protein expression, tyrosine phosphorylation, and kinase activity. As shown in Fig. 6A, total EphA2 protein is detectable in all eight human melanoma cell lines and 72 h treatment with 300 nmol/L dasatinib does not alter EphA2 protein expression levels. However, dasatinib inhibits EphA2 tyrosine phosphorylation in intact cells (Fig. 6B and C) as well as EphA2 kinase activity in an *in vitro* kinase activity assay using recombinant EphA2 protein (Fig. 6D). These data show that EphA2 is present in human melanoma cells and that EphA2 kinase activity is directly inhibited by dasatinib.

Discussion

SFKs participate in the regulation of many different biological processes, including cell adhesion, motility, invasion, differentiation, proliferation, and survival (9-17). The observation that SFKs can be overexpressed and overactivated in a wide variety of human cancers, and that this may be linked to the progression of human cancer, has made SFKs attractive molecular targets for therapeutic intervention. With the recent development of several clinically relevant inhibitors of SFKs, early-phase clinical trials with these drugs are currently under way. However, the effect of SFK inhibition in any given tumor type cannot be predicted precisely due to the myriad of roles of SFKs in controlling fundamental cellular processes. Here, we investigated the contribution of SFKs in human malignant melanoma cells using the small-molecule inhibitor of SFKs, dasatinib.

Malignant melanoma is a tumor characterized by the early formation of widespread metastases despite a comparably small size of the primary tumor. Multiple factors involved in invasion and metastasis of melanoma cells have been described (41, 42); however, little progress has been made in developing effective therapeutics to prevent metastatic spread of melanoma. In this report, we identify dasatinib as a potent inhibitor of melanoma cell migration and invasion at nanomolar concentrations (IC_{50} ≈50 nmol/L). Moreover, the inhibitory effect of dasatinib on motility of human melanoma cells is not due to growth arrest or apoptosis, as dasatinib does not markedly affect proliferation and survival of the eight human melanoma cell lines tested, even at micromolar concentrations. Dasatinib completely abolished the migration and invasion characteristics of A2058 and 1205-Lu cells at 300 nmol/L. These observations are consistent with earlier findings that showed little or no effect of dasatinib on proliferation and survival of prostate, pancreatic, and colon cancer cells (11, 43, 44). In contrast to this, other reports showed the ability of dasatinib to induce apoptosis in specific subsets of cell lines derived from other solid tumors, including sarcoma and lung (45, 46). Therefore, different cell types differentially depend on SFK kinase activity for survival. Also, because dasatinib is a multitargeted inhibitor, induction of apoptosis could also be attributed—at least in some cell lines and particularly at higher concentrations—to the inhibition of other targets.

Treatment of melanoma cells with nanomolar concentrations of dasatinib completely abolished SFK kinase activity as detected by antibody against the autophosphorylation site of c-Src (Tyr^{419}). Because this antibody cross-reacts with the autophosphorylation sites in other SFKs, we cannot exclude that SFKs other than c-Src are inhibited by dasatinib. Blockade of SFK activity also correlates with greatly reduced phosphorylation of its downstream substrates, FAK and Crk-associated

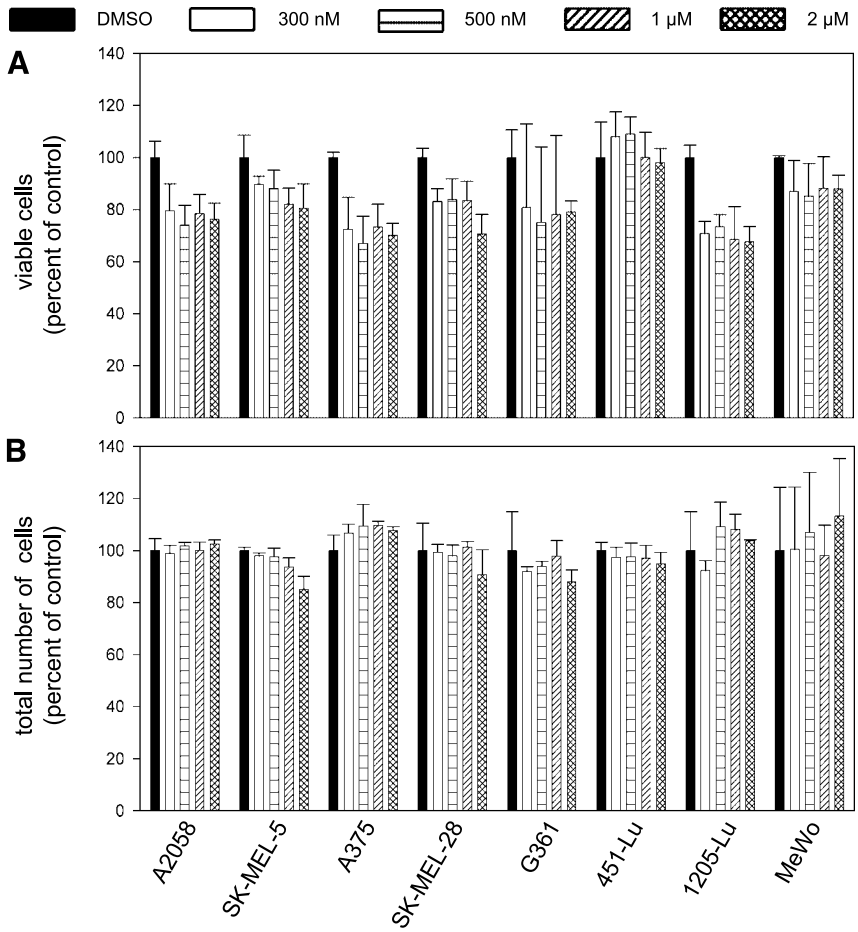

FIGURE 3. Dasatinib does not inhibit proliferation and viability of melanoma cells. Human melanoma cells (1,000 per well) were seeded in 96-well plates and exposed to increasing concentrations of dasatinib or DMSO vehicle control for 72 h (viability assay) or 96 h (proliferation assay). To determine viable cells (**A**), a tetrazolium compound was added to each well and the absorbance of the resulting formazan product was measured at 490 nm as per the supplier's instructions (Promega). To determine total cell number (**B**), cells were first lysed and total lactate dehydrogenase enzyme concentration was then measured as per the supplier's instructions (Promega). Percent of viable cells and percent of total cells (cell number) were normalized to DMSO-treated cells. Columns, average and SD of four individual measurements at each indicated concentration of dasatinib.

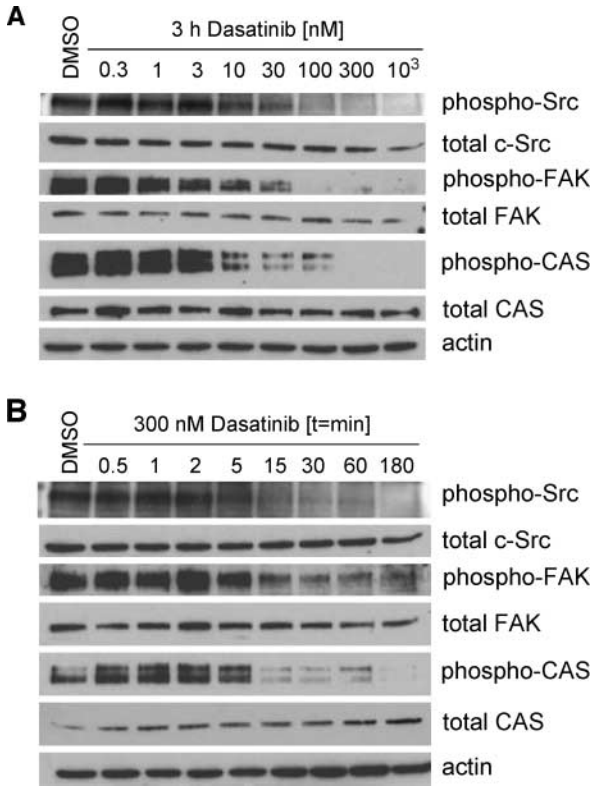

FIGURE 4. Dose- and time-dependent effects of dasatinib on melanoma cell signaling. To determine dose-dependent effects of dasatinib on signaling in human melanoma cells, A2058 cells were treated for 3 h with DMSO vehicle control or increasing concentrations of dasatinib as indicated (**A**). Three hundred nanomolar dasatinib was added to A2058 cells to determine time-dependent effects on cell signaling from 0.5 to 180 min as indicated (**B**). The levels of activated (phospho) and total Src, FAK, and $p130^{CAS}$ proteins were measured by Western blot analysis. Phospho-Src antibody detects SFKs phosphorylated at the equivalent of c-Src Tyr^{419}; phospho-FAK antibody detects phosphorylation of $Tyr^{576/577}$ in FAK; and phospho-CAS antibody detects phosphorylation of Tyr^{410} in $p130^{CAS}$.

substrate ($p130^{CAS}$), which are important in cell adhesion, migration, and invasion (27-29). Furthermore, the concentration of dasatinib needed to block migration and invasion of melanoma cells is similar to the concentration (<50 nmol/L) needed to block SFK/FAK/$p130^{CAS}$ signaling in seven of eight human melanoma cell lines. Moreover, dasatinib inhibits SFK/FAK/$p130^{CAS}$ phosphorylation events with similar kinetics (<15 min). Matrix metalloproteinase-9 (MMP-9) has previously been identified as a downstream target of SFK/FAK/$p130^{CAS}$ signaling (11). Consistent with this and with the critical role of MMP-9 in invasion, dasatinib blocks MMP-9 protein expression in A2058 human melanoma cells with an IC_{50} between 3 and 10 nmol/L. These findings suggest that the SFK/FAK/$p130^{CAS}$ signaling pathway plays an important role in the migration and invasion of melanoma cells. Because MMP-9 levels were too low or undetectable in other cell lines, it is possible that additional MMPs participate in SFK downstream signaling, too.

The EphA2 protein is a member of the Eph family of receptor tyrosine kinases that is overexpressed and/or overly active in several different types of cancer, including melanoma (35-40). We here show that dasatinib directly inhibits the kinase activity of EphA2, without affecting expression levels of total EphA2 protein. Although the precise roles of Eph receptors in general and of EphA2 in particular are not well understood, a study using EphA2 receptor variants that were either lacking the cytoplasmic domain or carrying a point mutation that inhibits its kinase activity resulted in decreased tumor volume and increased tumor apoptosis in a mouse model of breast cancer (37). In addition, the numbers of metastases were significantly reduced in both experimental and spontaneous metastasis models. The effects on growth and metastasis of the breast tumors expressing EphA2 signaling–defective mutants were not due to reduced angiogenesis, because the number of blood vessels was similar to that of wild-type tumors. Rather, tumor cells expressing the EphA2 mutants were defective in RhoA GTPase activation and cell migration (37).

Taken together, our findings suggest that dasatinib exerts its actions on human melanoma cells at least in part through blockade of major signaling pathways involved in cell migration and invasion, in particular the SFK/FAK/$p130^{CAS}$ and the EphA2 signaling pathway. Based on our results, SFK/FAK/$p130^{CAS}$ as well as EphA2 signaling may have critical roles in melanoma tumor progression.

Materials and Methods

Matrigel Invasion Assay

Invasion assays were done using 24-well BD BioCoat Matrigel Invasion Chambers as per the supplier's instructions (BD Biosciences). Briefly, 20,000 A2058 or 50,000 1205-Lu cells in 0.2% serum-containing medium were placed in the inserts. The wells were filled with supernatant from fully confluent grown A2058 or 1205-Lu cells (conditioned medium, 10% serum). Both chambers were immediately treated with dasatinib or DMSO control as indicated. Twenty-four hours later, noninvading cells were removed and the invaded cells were fixed and stained using Diff-Quik reagent. Using ×10 magnification, three photomicrographs of different areas were taken from each invasion chamber and the cell numbers were counted.

MMP-9 Protein Expression Assay

Total MMP-9 protein concentration in cell culture supernatant was determined using the MMP-9 Biotrak activity assay system as per the supplier's instructions (Amersham Biosciences). Briefly, 300,000 A2058 cells were seeded overnight in 10-cm round cell culture plates and then treated for 96 h with increasing amounts of dasatinib or DMSO control dissolved in 1% serum-containing medium. Supernatants were collected and analyzed in duplicates. This assay is based on a two-site ELISA sandwich format using two antibodies directed against different epitopes of MMP-9. During the first incubation step, MMP-9 present in the samples or the standards is bound to a microplate precoated with antibody. During the second incubation step, detection antibody conjugated to horseradish peroxidase is added, which forms an immobilized complex. The amount of peroxidase bound to each well is determined by the addition of tetramethylbenzidine. The reaction is stopped by the addition of an acid solution and the resultant color measured at 450 nm

in a microplate spectrophotometer. The concentration of proMMP-9 in samples is determined by interpolation from a standard curve.

Western Blotting and Immunoprecipitation

For Western blotting, cells were washed in buffer (1× PBS, 1 mmol/L Na-orthovanadate) and lysed in TGH buffer (1% Triton X-100, 10% glycerol, 50 mmol/L NaCl, 50 mmol/L HEPES, 1 mmol/L EGTA, 1% Na-deoxycholate, 1 mmol/L Na-orthovanadate, 2 μg/mL aprotinin, 0.5 μg/mL leupeptin, 50 μg/mL antipain, and 1 mmol/L phenylmethylsulfonyl fluoride). Total protein amount was determined using the Bio-Rad Protein Assay reagent and equal amounts of total protein were loaded in each lane of a 10% SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred to nitrocellulose membrane, washed with PBS/0.1% Tween 20, and incubated as per the suppliers' instructions in either 1× PBS/5% milk or 1× PBS/5% bovine serum albumin overnight with the first antibody. The membrane was then washed with PBS/0.1% Tween 20, incubated for 1 h at room temperature with alkaline phosphatase–linked antirabbit or antimouse secondary antibodies, and visualized using SuperSignal West Pico Reagent (Pierce). For detection of β-actin, the blot was incubated with stripping buffer [2% SDS, 64 mmol/L Tris (pH 6.7), 0.7% β-mercaptoethanol], reblotted with anti–β-actin (Sigma) for 1 h at room temperature, followed by incubation with alkaline phosphatase–linked antimouse secondary antibody.

Immunoprecipitation was done as per the supplier's instructions using 500 μg total protein and Ultralink immobilized protein A/G agarose (Pierce). Primary antibodies total-p38, phospho-p38 (Thr^{180}/Tyr^{182}), phospho-Src (Tyr^{419}), total-FAK, phospho-FAK (Tyr^{576}/Tyr^{577}), phospho-p130Cas (Tyr^{410}), total-p44/42 mitogen-activated protein kinase (Erk1 and Erk2), phospho-p44/42 mitogen-activated protein kinase (Erk1 and Erk2, Thr^{202}/Tyr^{204}), phospho-AKT (Ser^{473}), total-AKT, and phospho-Stat3 (Tyr^{705}) were all from Cell Signaling. Total-p130Cas (C-20) and total Stat3 (C-20) were from Santa Cruz Biotechnologies. Antibodies for total EphA2 (clone B2D6, IP; clone D7, WB) and total c-Src (GD11) were from Upstate.

Cell Lines and Cell Culture

All human melanoma cells were obtained from the American Type Culture Collection. MeWo, SK-Mel-5, SK-Mel-28, A375, A2058, and G361 cells were maintained in RPMI 1640 supplemented with 5% fetal bovine serum. 1205-Lu and 451-Lu cells were grown in tumor cell medium (three parts MCDB153 medium, one part Leibovitz's L-15 medium) supplemented with 2.5% fetal bovine serum and 5 μg/mL human insulin. All media were supplemented with 50 units/mL penicillin and 50 μg/mL streptomycin.

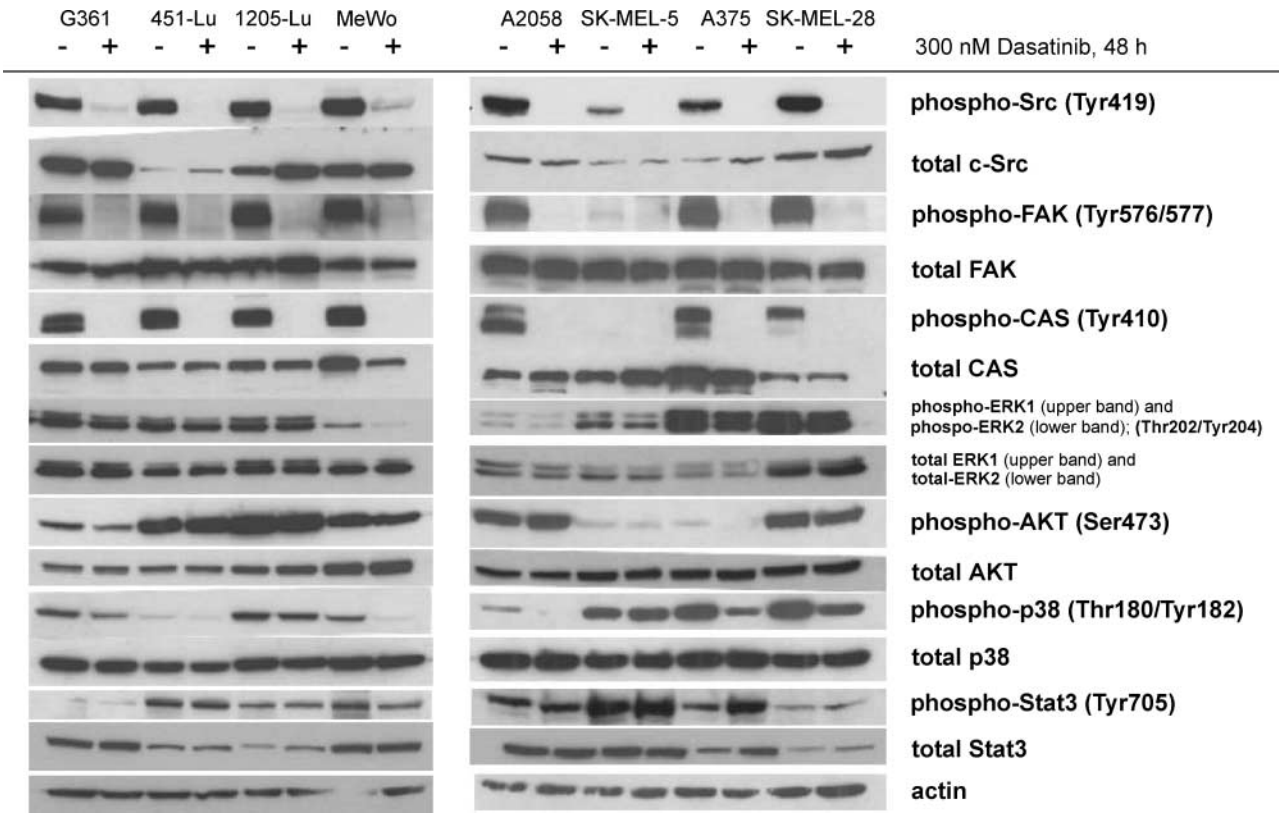

FIGURE 5. Signaling profile of melanoma panel treated with dasatinib. To determine the effects of dasatinib on cell signaling in human melanoma, eight human melanoma cell lines were treated for 24 h with 300 nmol/L dasatinib or DMSO vehicle control. The levels of activated (phospho) and total c-Src, FAK, $p130^{CAS}$, Erk1 (p44 mitogen-activated protein kinase), Erk2 (p42 mitogen-activated protein kinase), AKT, p38, and Stat3 proteins were measured by Western blot analysis as described in Materials and Methods.

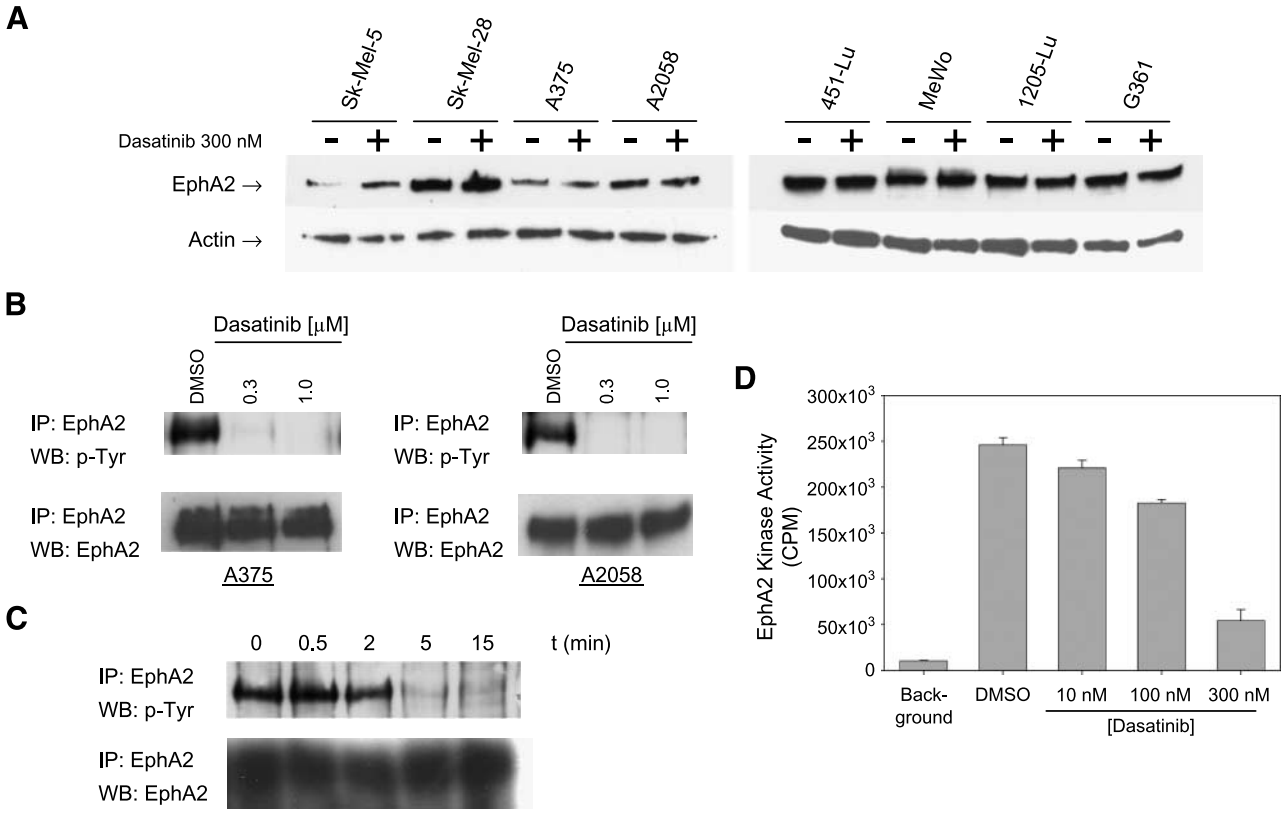

FIGURE 6. Dasatinib directly inhibits EphA2 kinase activity. Total EphA2 protein expression of eight human melanoma cell lines before and after treatment for 72 h with 300 nmol/L dasatinib (**A**) was determined by Western blotting. EphA2 tyrosine phosphorylation in A375 and A2058 cells treated with dasatinib for 24 h was determined by immunoprecipitation (*IP*) with EphA2 (clone B2D6) antibodies followed by Western blotting (*WB*) with antiphosphotyrosine (p-Tyr) antibodies and anti EphA2 (clone D7; **B**). Time-dependent effect of 300 nmol/L dasatinib on EphA2 tyrosine phosphorylation in A2058 melanoma cells (**C**). Dose-dependent effect of dasatinib on recombinant EphA2 protein kinase activity *in vitro* (**D**).

Viability and Proliferation Assays

Cells (1,000 per well) were seeded in 96-well plates and allowed to adhere overnight. The next day, dasatinib or DMSO vehicle control was added to the cells as indicated and the cells were incubated for another 72 h (viability assay) or 96 h (proliferation assay) in the presence of 5% (RPMI) or 2.5% (tumor cell medium) serum as described above. For viability assays, cells were directly incubated with the MTS substrate (CellTiter-96 Aqueous One Solution Cell Proliferation Assay, Promega). For proliferation assays, cells were first lysed and the supernatant was incubated with lactate dehydrogenase detection reagent (CytoTox-96 Non-Radioactive Cytotoxicity Assay, Promega). For both assays, absorbance was measured at 490 nm as per the supplier's instructions and percent viability and cell number (proliferation) was normalized to the absorbance of DMSO-treated cells. For each cell line and treatment, the absorbance values of at least three wells were used to analyze the data. For statistical analysis of the data used to generate Fig. 3, we compared DMSO-treated cells versus different concentrations of dasatinib-treated cells and used a two-tailed, paired t test. None of the comparisons showed a significant difference ($P > 0.05$).

Migration Assays

"Wounds" were made by scratching a pipette tip on confluent cells cultured in 12-well dishes. Cells were then incubated with dasatinib or DMSO vehicle control as indicated. Migration of cells into the wound was photographed at ×10 magnification under a microscope.

EphA2 Kinase Assays

Recombinant EphA2 was obtained from Upstate Cell Signaling Solutions. *In vitro* kinase activity assays of EphA2 were done as per the supplier's instructions. Briefly, recombinant EphA2 protein was preincubated with increasing concentrations of dasatinib or DMSO, followed by addition of [γ-33P]ATP and substrate, poly-(Glu4-Tyr). The level of substrate phosphorylation was quantified in a scintillation counter.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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