Action of the Src Family Kinase Inhibitor, Dasatinib (BMS-354825), on Human Prostate Cancer Cells

Sangkil Nam,[1] Donghwa Kim,[3] Jin Q. Cheng,[3] Shumin Zhang,[1] Ji-Hyun Lee,[2] Ralf Buettner,[1] Janni Mirosevich,[1] Francis Y. Lee,[4] and Richard Jove[1,3]

[1]Molecular Oncology and [2]Biostatistics Programs, H. Lee Moffitt Cancer Center and Research Institute, [3]Department of Pathology, University of South Florida College of Medicine, Tampa, Florida; and [4]Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey

Abstract

Src family kinases (SFK) are currently being investigated as targets for treatment strategies in various cancers. The novel SFK/Abl inhibitor, dasatinib (BMS-354825), is a promising therapeutic agent with oral bioavailability. Dasatinib has been shown to inhibit growth of Bcr-Abl–dependent chronic myeloid leukemia xenografts in nude mice. Dasatinib also has been shown to have activity against cultured human prostate and breast cancer cells. However, the molecular mechanism by which dasatinib acts on epithelial tumor cells remains unknown. In this study, we show that dasatinib blocks the kinase activities of the SFKs, Lyn and Src, in human prostate cancer cells at low nanomolar concentrations. Moreover, focal adhesion kinase and Crk-associated substrate ($p130^{CAS}$) signaling downstream of SFKs are also inhibited at similar concentrations of dasatinib. Consistent with inhibition of these signaling pathways, dasatinib suppresses cell adhesion, migration, and invasion of prostate cancer cells at low nanomolar concentrations. Therefore, dasatinib has potential as a therapeutic agent for metastatic prostate cancers harboring activated SFK and focal adhesion kinase signaling. (Cancer Res 2005; 65(20): 9185-9)

Introduction

Elevated levels and activities of Src family kinases (SFK), including Src and Lyn, have been shown in numerous human cancer cell lines and tumor tissues (1–3). SFKs phosphorylate tyrosyl residues of critical cellular substrates, resulting in the activation of oncogenic signal transduction pathways (4, 5). One such substrate of SFKs, focal adhesion kinase (FAK), has an important role in integrin signaling (6) and is highly expressed in many tumor cells including prostate cancer (7, 8). Tyrosyl phosphorylation of FAK stimulates its ability to modulate cell adhesion, migration, and invasion (6). In addition, tyrosyl phosphorylation of Crk-associated substrate ($p130^{CAS}$), another substrate of SFKs, is important for cell motility and invasion (9, 10). Originally, dasatinib (BMS-354825) compound [*N*-(2-chloro-6-methylphenyl)-2-(6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide] was selected as a synthetic small-molecule inhibitor of SFKs (11). In addition, dasatinib was subsequently found to be a Bcr-Abl kinase inhibitor, as has been shown earlier for other SFK inhibitors (12, 13). Furthermore, a recent phase I clinical trial determined that dasatinib is a promising agent for treatment of chronic myelogenous leukemia with activated Bcr-Abl kinase. Dasatinib also showed activity against epithelial tumor cells, including human prostate and breast cancer cells (11). However, the molecular mechanisms of dasatinib's action on epithelial tumor cells remain unknown. Here, we report that dasatinib inhibits SFK/FAK/$p130^{CAS}$ signaling at low nanomolar concentrations, associated with inhibition of cell adhesion, migration, and invasion of human prostate cancer cells.

Materials and Methods

Cells and reagents. DU-145 and LNCaP prostate cancer cells were obtained from American Type Culture Collection and cultured in RPMI 1640 containing 10% fetal bovine serum. Polyclonal antibodies to the phosphoproteins p-Src (Tyr416), p-FAK (Tyr576/577), p-$p130^{CAS}$ (Tyr410), p-Stat3 (Tyr705), p-ERK1/2 (Thr202/Tyr204), and p-AKT (Ser473) were obtained from Cell Signaling Technologies (Cambridge, MA). Polyclonal antibodies to Lyn, FAK, and $p130^{CAS}$ proteins were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to the phosphoprotein p-FAK (Tyr397 and Tyr861) were from BioSource International (Camarillo, CA). Monoclonal antibody to c-Src was obtained from Upstate Biotechnology (Lake Placid, NY). Dasatinib was obtained from Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ). Dasatinib was synthesized by the addition of methylpyrimidine to the 2-amino group of thiazole, followed by reaction with hydroxyethyl piperazine (11).

Lyn and Src kinase assays *in vitro*. Lyn and Src kinase assays were done *in vitro* as described in the supplier's protocol (Upstate Biotechnology). Twenty-microliter aliquots of each 25 μL reaction mixture were transferred onto the center of substrate-binding phosphocellulose paper squares. Assay squares were washed thrice with 0.75% phosphoric acid, and transferred to vials with 5 mL of scintillation cocktail. After quantifying activity with a LS6500 Scintillation Counter (Beckman Coulter, Fullerton, CA), radioactivity in assay squares was directly visualized by autoradiography.

Western blot analysis. Western blot analysis was done as previously described (14). Primary phosphospecific antibodies were incubated in TBS (pH 7.5) with 0.1% Tween 20 and 5% bovine serum albumin with gentle agitation overnight at 4°C. horseradish peroxidase–conjugated secondary antibodies were diluted in TBS or PBS with 5% nonfat milk and incubated for 1 hour at room temperature. Positive immunoreactions were detected using the Chemiluminescent Substrate system (Pierce, Rockford, IL). For immunoprecipitation of Lyn, cell lysates (300 μg) were incubated with Lyn antibody overnight at 4°C, followed by protein A/G-agarose for 1 hour at 4°C (Pierce). Samples were immunoblotted with p-Src family antibody (Tyr416), which cross-reacts with p-Lyn (Tyr396).

Cell adhesion assay. Cells (1×10^5/well) were pretreated with dasatinib for 30 minutes and adhered onto fibronectin-coated 96-well plates (BD Sciences, San Jose, CA) for 1 hour at 37°C. All procedures were done as described previously (15). Briefly, nonadhered cells were removed by washing thrice with serum-free medium. Attached cells were fixed with 70%

Requests for reprints: Richard Jove, Beckman Research Institute, City of Hope National Medical Center, 1500 East Duarte Rd, Duarte, CA 91010. Phone: 626-256-4673; Fax: 626-256-8708; E-mail: rjove@coh.org.

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methanol for 10 minutes. Cells were stained with 0.02% crystal violet in 0.2% ethanol solution and dissolved with 100 μL Sorenson solution/well. Plates were analyzed with an automated ELISA reader at 540 nm. Each experiment was done in triplicate.

Wound healing assay for cell migration. Monolayer wounds were made using a pipette tip on confluent DU-145 cells cultured in six-well plates. Cells were treated with dasatinib or DMSO as vehicle control in a dose-dependent manner and then allowed to migrate into the denuded area for 6 hours. Cell migration was visualized at 10× magnification using a TE 2000 Inverted Fluorescence Microscope (Nikon, Melville, NY) and IPLab 3.6 software (Scanalytics, Fairfax, VA), and photographed with a Retiga 1300 CCD Camera (Qimaging, Burnaby, B.C., Canada). Distances of denuded areas were measured as pixel units.

Analysis of matrix metalloproteinase-9 activity. Matrix metalloproteinase-9 (MMP-9) activity assays were done with a MMP-9 human ELISA kit (Amersham Biosciences, Piscataway, NJ). Cells were washed with PBS twice and serum-free medium twice. Fresh serum-free medium was added to cell cultures and cells were exposed to dasatinib or DMSO as vehicle control for 24 hours. Cell culture medium was collected and concentrated using Centrifugal Ultra Filters (Millipore, Billerica, MA). Normalized proteins (2 μg/well) were added to 96-well ELISA plates. The reaction was stopped with 100 μL of 1 mol/L sulfuric acid and absorbance was measured with an automated ELISA plate reader at 450 nm. Each experiment was done in quadruplicate.

Cell invasion assay. Cell invasion assays were done on polycarbonate membrane inserts (8 um pore size; Chemicon International, Temecula, CA). DU-145 cells were washed with PBS once and serum-free medium twice. Cells were resuspended with fresh serum-free medium and dasatinib or DMSO as vehicle control was added. Cells (5×10^5/well) in 300 μL of serum-free medium were placed over the inner chamber of the insert in a 24-well tissue culture plate, and 500 μL of serum-free medium was placed in the outer chamber of the insert. The plates were incubated for 24 hours at 37°C. After 24 hours, the cells that migrated through to the lower surface of the extracellular matrix layer were stained and dissolved in 10% acetic acid. Solutions were transferred to a 96-well plate and absorbances measured with an automated ELISA plate reader at 540 nm. Each experiment was done in triplicate.

Statistical analysis. Descriptive statistics, such as mean values and SD, were calculated for the biological effects of dasatinib (i.e., inhibition of cell adhesion, MMP-9 activity, and invasion) by dose levels (nmol/L). To determine statistical significance between pair-wise dose levels, the exact Wilcoxon two-sample test was used, considering the small sample sizes. One-sided tests at a significance level of 0.05 were examined. All data were analyzed using the SAS software (version 9.1, SAS Institute, Cary, NC).

Results and Discussion

Dasatinib inhibits Src family kinase activity *in vitro* and *in vivo*. The human DU-145 cell line is an androgen-independent, highly aggressive metastatic prostate cancer cell line, whereas the androgen-dependent human LNCaP prostate cancer cell line exhibits relatively low aggressiveness and low metastatic potential (16). A previous study reported that Lyn is expressed in a majority of primary human prostate cancers and thus might be a potential target protein for treatment of this disease (17). Dasatinib is a thiazole- and pyrimidine-based SFK/Abl kinase inhibitor (Fig. 1*A*). To confirm that dasatinib (BMS-354825) directly inhibits Lyn and Src kinase activity, *in vitro* kinase assays were done with active recombinant Lyn or Src proteins. Dasatinib showed an inhibitory effect on both Lyn and Src kinase activities with IC_{50} values of 8.5 and 3.0 nmol/L, respectively (Fig. 1*B* and *C*).

Western blot analysis was used to evaluate total and autophosphorylated Lyn and Src protein levels in intact DU-145 and LNCaP cells. DU-145 cells expressed higher levels of Lyn than LNCaP cells,

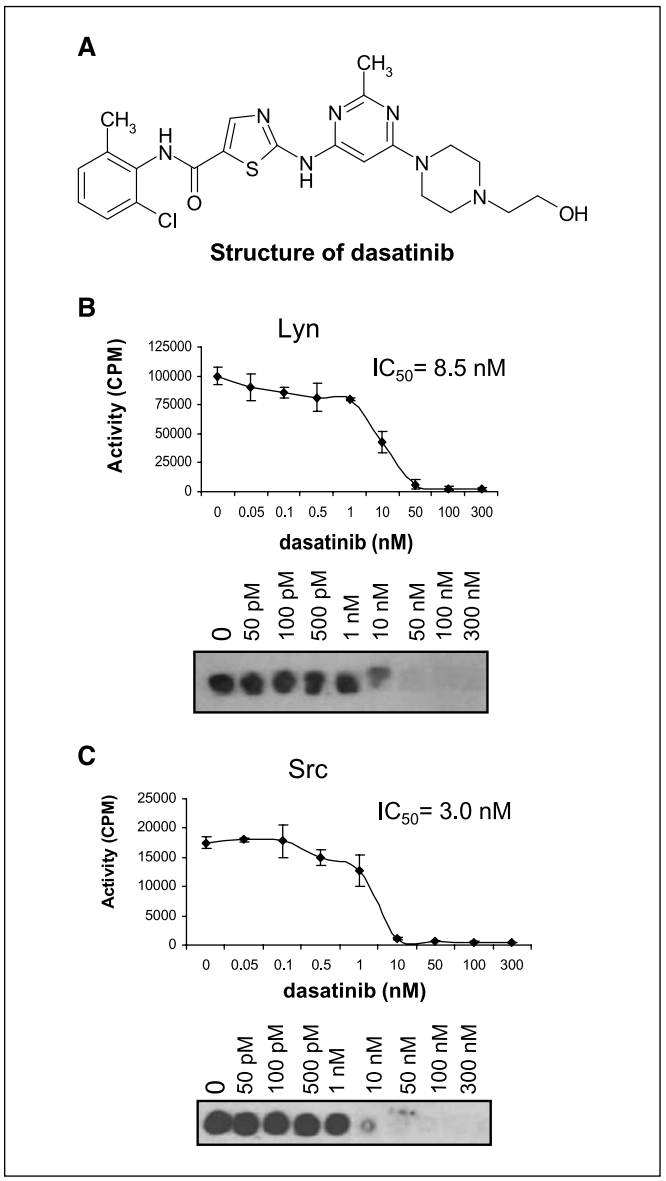


Figure 1. Dasatinib inhibits Lyn and Src kinase activities *in vitro*. *A,* the chemical structure of dasatinib. *B,* for the *in vitro* Lyn kinase assay, 20 ng of recombinant Lyn and 0.1 mg/mL poly (Glu4-Tyr) substrate were preincubated in the presence or absence of various dasatinib concentrations for 10 minutes. [γ-^{32}P]ATP (5 μCi) was added per 25 μL reaction mixture for 15 minutes. Radiolabeled reaction mixtures were transferred to substrate-binding phosphocellulose. *C,* for the *in vitro* Src kinase assay, 20 ng of recombinant Src and 250 μmol/L of synthetic Src substrate peptide were preincubated in the presence or absence of various dasatinib concentrations for 10 minutes. [γ-^{32}P]ATP (10 μCi) were added per 25 μL reaction mixture for 15 minutes. Radiolabeled reaction mixtures were transferred to substrate-binding phosphocellulose. Radiolabeled substrates from both *in vitro* kinase assays were counted with a scintillation counter to determine IC_{50} values of enzyme activity and visualized by autoradiography. *Points,* mean; *bars,* ± SD (n = 3).

whereas LNCaP cells had higher levels of Src than DU-145 cells (Fig. 2*A* and *C, bottom*). Dasatinib caused a substantial decrease of autophosphorylated p-Lyn and p-Src at 100 nmol/L in both cell lines 6 hours after treatment (Fig. 2*A* and *C, top*). Time course studies showed that the levels of p-Lyn and p-Src were rapidly decreased as early as 30 minutes after 100 nmol/L dasatinib treatment in DU-145 cells (Fig. 2*B* and *D, top*). A dose-dependent study using whole lysates of cells treated with dasatinib for 6 hours

showed a large decrease in p-Lyn and p-Src levels at 1 to 10 nmol/L in DU-145 cells (Fig. 2*B* and *D, bottom*).

Dasatinib selectively inhibits focal adhesion kinase and p130^{CAS} signaling. FAK is a nonreceptor tyrosine kinase and increased levels of expression and tyrosyl phosphorylation of FAK have been shown in epithelial tumors (8). In this signaling pathway, the autophosphorylation of FAK at Tyr397 recruits SFKs that phosphorylate FAK at Tyr576, Tyr577, and Tyr861 (6). Phosphorylation of these latter sites by SFKs is important for FAK downstream signaling (6). To assess whether dasatinib inhibits SFK/FAK signaling, DU-145 and LNCaP cells were exposed to dasatinib for 6 hours. Dasatinib almost totally abolished the levels of p-FAK at Tyr576/577 in DU-145 cells, whereas p-FAK was not detected in LNCaP cells (Fig. 3*A, top*) even though both cell lines expressed

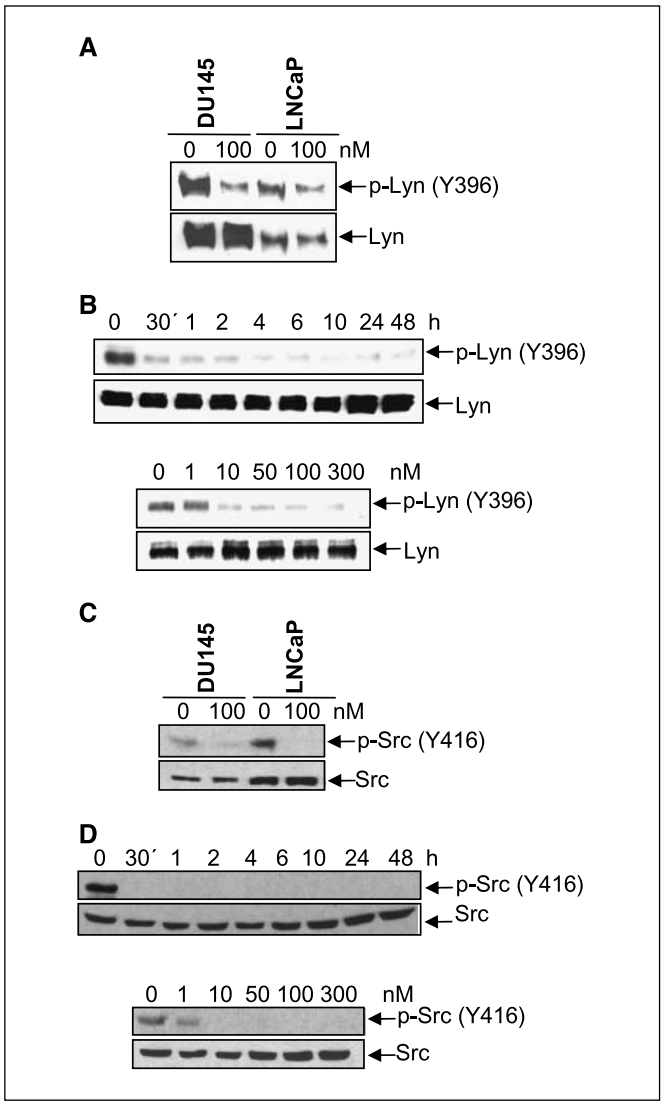


Figure 2. Dasatinib blocks tyrosyl phosphorylation of Lyn and Src in whole cells. *A* and *C,* DU-145 and LNCaP cells were treated with 100 nmol/L dasatinib for 6 hours. For p-Lyn detection, lysates were immunoprecipitated with Lyn antibody and blotted with p-Src family antibody (Tyr416), which cross-reacts with autophosphorylated p-Lyn (Tyr396). Western blot analysis was also done with specific antibodies to p-Src and Src. *B* and *D,* DU-145 cells were treated with 100 nmol/L dasatinib in a time-dependent manner (*top*) or for 6 hours in a dose-dependent manner (*bottom*). Western blot analysis was done as described above. DMSO was used as vehicle control for all experiments.

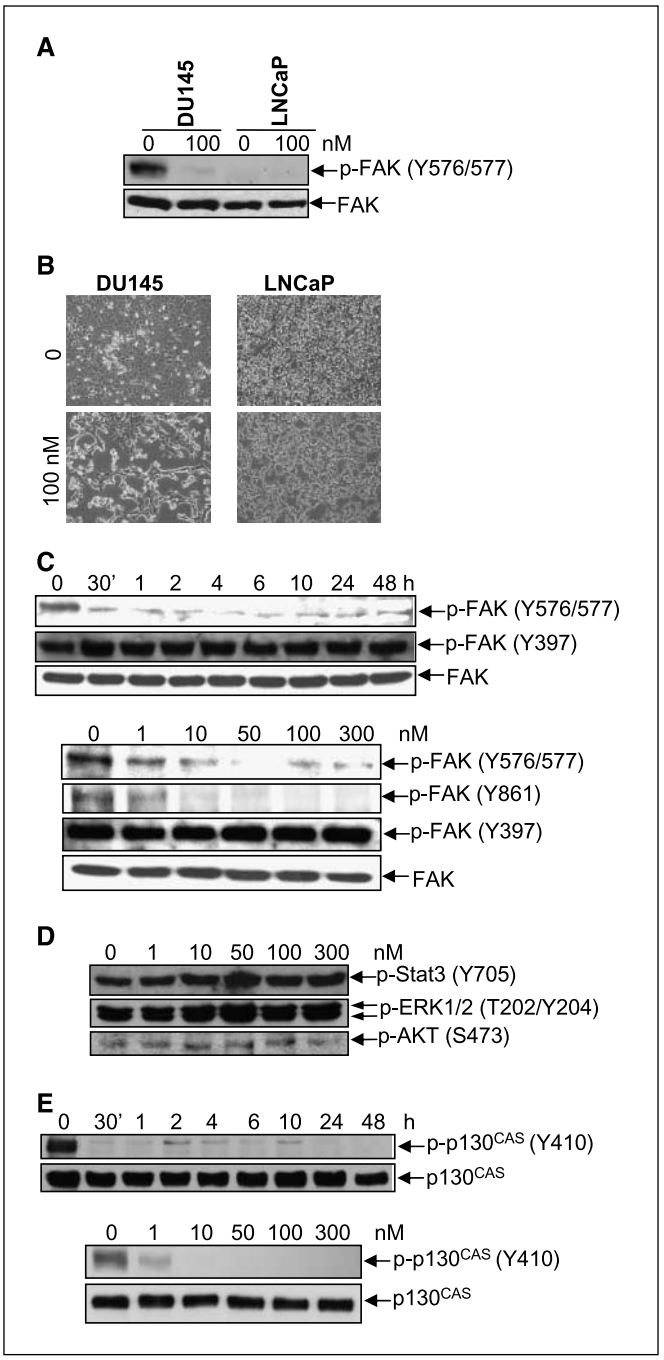
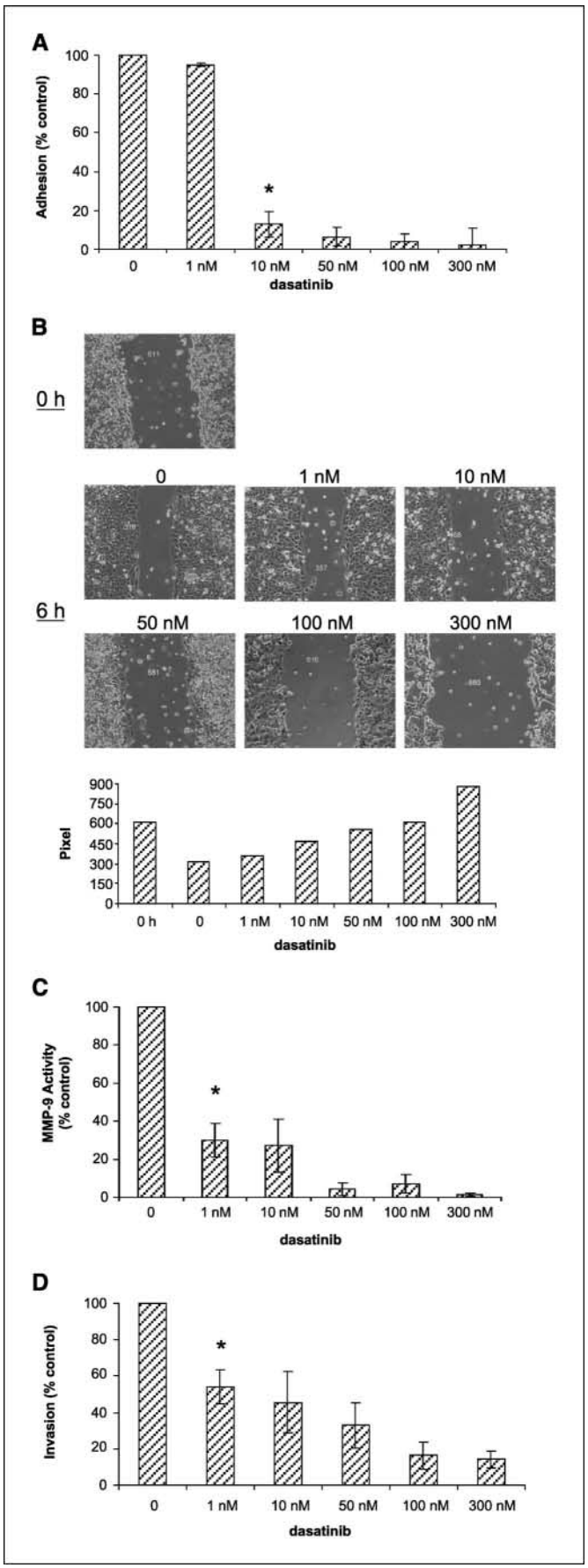


Figure 3. Effects of dasatinib on phosphorylation of signaling proteins downstream of SFKs. *A,* dasatinib blocks tyrosyl phosphorylation of FAK, but does not affect levels of total FAK in cells. DU-145 and LNCaP cells were treated with 100 nmol/L dasatinib for 6 hours. *B,* dasatinib decreases cell-to-cell contacts. DU-145 and LNCaP cells were treated with 100 nmol/L dasatinib for 24 hours. Decrease of cell-to-cell contact was visualized at 10× magnification by light microscopy. *C,* dasatinib blocks tyrosyl phosphorylation of FAK at Y576/577 and Y861, but not at Y397. DU-145 cells were treated with 100 nmol/L dasatinib in a time-dependent manner (*top*) or for 6 hours in a dose-dependent manner (*bottom*). Cell-free extracts were immunoblotted with specific antibodies to p-FAK at Tyr397, Tyr576/577, and Tyr861, and total FAK. *D,* dasatinib does not block phosphorylation of Stat3, ERK1/2, and AKT in cells. DU-145 cells were treated with dasatinib for 6 hours in a dose-dependent manner. Cell-free extracts were immunoblotted with specific antibodies to p-Stat3, p-ERK1/2, and p-AKT. *E,* dasatinib blocks tyrosyl phosphorylation of p130^{CAS}, but does not affect levels of total p130^{CAS}. DU-145 cells were treated with 100 nmol/L dasatinib in a time-dependent manner (*top*) or for 6 hours in a dose-dependent manner (*bottom*). Cell-free extracts were immunoblotted with specific antibody to p-p130^{CAS} (Tyr410) and p130^{CAS}.



similar levels of total FAK protein (Fig. 3*A, bottom*). Treatment with 100 nmol/L dasatinib for 24 hours had no effect on cell viability and total cell numbers, although partial inhibition of cell proliferation due to G_1 arrest was observed at 48 and 72 hours (data not shown). Most strikingly, however, there was a substantial loss of cell-to-cell contact in DU-145 cells (Fig. 3*B*). This effect may be related to the decrease in levels of p-FAK and p-p130^{CAS} (see below).

A time course study showed that 100 nmol/L dasatinib reduced the levels of p-FAK at Y576/577/861 in 30 minutes, whereas it did not change levels of p-FAK autophosphorylation at Tyr397 (Fig. 3*C, top*). A dose-dependent study also showed that cells treated with dasatinib for 6 hours exhibited decreased the levels of p-FAK at Tyr576/577/861 at 1 to 10 nmol/L, whereas the levels of p-FAK autophosphorylation at Tyr397 did not change (Fig. 3*C, bottom*). The reduced levels of p-FAK at Tyr576/577/861 correlated with a decrease in the levels of p-Lyn and p-Src (compare with Fig. 2*B* and *D*), suggesting that dasatinib targets SFK/FAK signaling. However, dasatinib does not directly inhibit FAK autophosphorylation at Tyr397 in DU-145 cells, consistent with a previous report that dasatinib did not inhibit FAK activity in an *in vitro* kinase assay (11).

Next, we examined whether dasatinib could reduce the phosphorylation levels of proteins involved in other tyrosine kinase signaling pathways. Levels of p-Stat3, p-Erk1/2, and p-Akt were not altered with dasatinib treatment (Fig. 3*D*). Notably, dasatinib does not induce apoptosis in DU-145 cells (data not shown), consistent with the lack of effect on levels of p-Akt and p-Stat3, both signaling proteins that are involved in tumor cell survival (18). Thus, our observations suggest that dasatinib predominantly acts by inhibiting SFK/FAK signaling in prostate cancer cells.

The p130^{CAS} protein is involved in integrin-mediated cell signaling and its SH3 domain interacts with FAK to form a FAK-p130 complex (6, 9). Tyrosyl phosphorylation of FAK at Tyr861 regulates its interaction with p130^{CAS} (19) and tyrosyl phosphorylation of p130^{CAS} is involved in cell motility and invasion (9, 10, 20). To determine whether inhibition of SFKs by dasatinib reduces levels of p-p130^{CAS}, DU-145 cells were exposed to dasatinib for time course and dose-dependent studies. Consistent with the inhibition

Figure 4. Dasatinib inhibits cell motility and invasion. *A,* dasatinib inhibits cell adhesion on fibronectin. DU-145 cells were pretreated with dasatinib for 30 minutes and adhered onto fibronectin-coated 96-well plates. Unattached cells were washed thrice with serum-free medium. Plates were read with an automated ELISA reader at 540 nm. *Columns,* mean; *bars,* ± SD; *, $P = 0.028$ ($n = 3$). *B,* dasatinib inhibits cell migration. Wound healing assay was done to determine whether dasatinib inhibits cell migration. After treatment with various dasatinib concentrations for 6 hours, cells were allowed to migrate into the denuded area for 6 hours. Cell migration was visualized at 10× magnification by light microscopy, and photographed with a digital camera. Width of denuded areas versus dasatinib concentration was plotted in pixels (*bottom graph*). *C,* dasatinib inhibits MMP-9 activity. DU-145 cells were washed with 1× PBS twice and serum-free medium twice. Fresh serum-free medium was added to plates and cells were exposed to dasatinib for 24 hours. Culture medium was collected and concentrated with an ultra-centrifugal filter device. Normalized proteins were added to 96-well ELISA plates. Reactions were stopped with 100 μL of 1 mol/L sulfuric acid and absorbances read with an automated ELISA plate reader at 450 nm. *Columns,* mean; *bars,* ± SD; *, $P = 0.029$ ($n = 4$). *D,* dasatinib inhibits cell invasion. DU145 cells in 300 μL of serum-free medium were placed over the inner chamber of the insert in a 24-well tissue culture plate, and 500 μL of serum-free medium was placed in the outer chamber of the insert. The plates were incubated for 24 hours at 37°C and 5% CO_2 atmosphere. After 24 hours, the invasive cells that migrated through the lower surface of the extracellular matrix layer were stained. The stained cells were dissolved in 10% acetic acid and absorbance was read with an automated ELISA reader at 540 nm. Each experiment was done in triplicate. *Columns,* mean; *bars,* ± SD; *, $P = 0.050$ ($n = 3$).

of p-FAK levels (Fig. 3*C*), dasatinib decreased p-p130^{CAS} levels, whereas total levels of p130^{CAS} were unaltered (Fig. 3*E*). The observed reduction of p-p130^{CAS} levels also correlates well with the reduction of p-Lyn and p-Src levels (Fig. 2*B* and *D*).

Dasatinib inhibits cell adhesion, migration, and invasion. FAK has an important role in the integrin signaling cascade (6). Cellular adhesion to extracellular matrix proteins such as fibronectin require integrins. To assess whether dasatinib blocks cell adhesion to the extracellular matrix, DU-145 cells were pretreated with the drug for 30 minutes to inhibit levels of p-FAK and p-p130^{CAS}. Cell adhesion to fibronectin was significantly inhibited by 10 nmol/L of dasatinib (Fig. 4*A*). This finding correlates with the observed reduction of p-FAK and p-p130^{CAS} (Fig. 3*C* and *E*), suggesting that the effects of dasatinib on p-FAK and p-p130^{CAS} levels contribute to inhibition of cell adhesion to fibronectin. In addition, wound-healing assays were done to determine whether dasatinib affects cell migration. Dasatinib inhibited cell migration at 1 to 10 nmol/L after 6 hours of treatment (Fig. 4*B*). These findings indicate that the reduction of p-FAK and p-p130^{CAS} levels by dasatinib is associated with inhibition of cell adhesion and migration.

A previous study showed that the inhibition of FAK reduced MMP-9 secretion (7). To determine whether down-regulation of p-FAK by dasatinib contributes to inhibition of MMP-9 activity, a MMP-9 activity assay was done with proteins secreted by cells treated with dasatinib for 24 hours. As shown in Fig. 4*C*, dasatinib significantly inhibited secretion of MMP-9 activity at 1 to 10 nmol/L. To assess whether inhibition of MMP-9 activity correlates with inhibition of cell invasion, DU-145 cells pretreated with dasatinib for 30 minutes were added onto the extracellular matrix layer of the assay chamber for 24 hours. Dasatinib significantly inhibited cell invasion at 1 to 10 nmol/L (Fig. 4*D*), consistent with a decrease in MMP-9 activity. These findings suggest that the reduction of p-FAK and p-p130^{CAS} levels by dasatinib may inhibit MMP-9 activity, resulting in decreased cell invasion.

Therapeutic implications. Dasatinib is an orally bioavailable and promising antitumor therapeutic agent for chronic myelogenous leukemia (11, 13). Recently, phase I clinical trials showed the efficacy of dasatinib for the treatment of chronic myelogenous leukemia with activated Bcr-Abl kinase.[5] In addition, dasatinib has been advanced into clinical trials for human epithelial solid tumors.[5] Many invasive epithelial tumors exhibit elevated SFK and FAK expression levels and activities (1–6). In this report, we show that dasatinib blocks SFK/FAK/p130^{CAS} signaling, resulting in inhibition of cell adhesion, migration, and invasion in prostate cancer cells. Based on this action of dasatinib on prostate cancer cells, we suggest that dasatinib has potential as an antitumor therapeutic agent in metastatic prostate cancers harboring activated SFK and FAK signaling.

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