

SKI-606 (bosutinib), a novel Src kinase inhibitor, suppresses migration and invasion of human breast cancer cells

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Abstract

Src family kinase activity is elevated in many human tumors, including breast cancer, and is often associated with aggressive disease. We examined the effects of SKI-606 (bosutinib), a selective Src family kinase inhibitor, on human cancer cells derived from breast cancer patients to assess its potential for breast cancer treatment. Our results show that SKI-606 caused a decrease in cell motility and invasion of breast cancer cell lines with an IC₅₀ of ~250 nmol/L, which was also the IC₅₀ for inhibition of cellular Src kinase activity in intact tumor cells. These changes were accompanied by an increase in cell-to-cell adhesion and membrane localization of β -catenin. By contrast, cell proliferation and survival were unaffected by SKI-606 at concentrations sufficient to block cell migration and invasion. Analysis of downstream effectors of Src revealed that SKI-606 inhibits the phosphorylation of focal adhesion kinase (FAK), proline-rich tyrosine kinase 2 (Pyk2), and Crk-associated substrate (p130^{Cas}), with an IC₅₀ similar to inhibition of cellular Src kinase. Our findings indicate that SKI-606 inhibits signaling pathways involved in controlling tumor cell motility and invasion, suggesting that SKI-606 is a promising therapeutic for breast cancer. [Mol Cancer Ther 2008;7(5):1185–94]

Introduction

The cellular Src (c-Src) protein is a nonreceptor tyrosine kinase normally maintained in an inactive conformation via

intramolecular interactions. When acted upon by upstream signals such as growth factors, c-Src undergoes a conformational change resulting in activation of its kinase (1, 2). Importantly, c-Src coordinates multiple signaling pathways known to be involved in tumor progression, such as proliferation, survival, motility, angiogenesis, cell-cell communication, adhesion, and invasion (3, 4). Therefore, c-Src is a potential molecular target for therapy of human neoplasias, including breast cancer. The recent introduction of Src family kinase inhibitors in clinical trials for solid tumors necessitates a better understanding of their mechanism of action to optimize their clinical effectiveness in patients.

Early studies reported elevated levels of c-Src tyrosine kinase activity in breast cancer samples when compared with normal tissue (5). These findings were substantiated using immunohistochemistry, *in vitro* kinase assays, and Western blot analyses (6–8). Previously, we have shown that Src is significantly activated in invasive carcinoma compared with paired nonneoplastic parenchyma from 45 patients with stage II breast cancer ($P < 0.001$; ref. 9). The mechanisms underlying Src kinase activation in breast cancer are not fully elucidated yet, but evidence points to the overexpression or altered activity of upstream receptors such as epidermal growth factor receptor, Her2/neu, platelet-derived growth factor receptor, fibroblast growth factor receptor, c-Met, integrins, and steroid hormone receptors (2, 10, 11). Elevated levels of protein tyrosine phosphatase 1B (PTP1B) may also contribute to high c-Src kinase activity in breast cancer by dephosphorylating c-Src on its negative regulatory domain (12).

Multiple studies using various Src kinase inhibitors and dominant-negative mutants support the finding that inhibiting c-Src activity in a variety of tumor sites blocks cell proliferation, induces apoptosis, and decreases metastatic potential, thereby implicating c-Src as an attractive molecular target for anticancer therapy (13–16). Given the poor survival rates of patients with distant breast cancer metastases (17) and the association of c-Src activity with aggressive neoplastic behavior, development of Src inhibitors for cancer treatment is of considerable interest. SKI-606 (bosutinib) is a potent, orally bioavailable, dual Src/Abl kinase inhibitor previously shown to have antiproliferative effects in chronic myelogenous leukemia cells, to inhibit colon tumor cell colony formation in soft agar, and to suppress tumor growth in K562 and colon tumor cell xenograft models (18, 19).

We report here that in human cancer cells derived from breast cancer patients, SKI-606 preferentially inhibits cell spreading, migration, and invasion, while leading to stabilized cell-to-cell adhesions and membrane localization

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of β -catenin. These effects are not associated with changes in proliferation or survival and are accompanied by inhibition of the Src/focal adhesion kinase (FAK)/Crk-associated substrate (p130^{Cas}) signaling pathway. Taken together, our data point to SKI-606 as a promising anti-invasive and antimetastatic drug for the potential treatment of breast cancer.

Materials and Methods

Cell Lines and Reagents

All human cancer cell lines [MDA-MB-468, MDA-MB-231, MCF-7, and MDA-MB-435s (isolated from a breast cancer patient yet melanoma derived)] were obtained from the American Type Culture Collection (ATCC) and cultured following ATCC protocols. Src, Yes, and Fyn knockout mouse embryo fibroblasts (SYF^{-/-}) and SYF^{-/-} cells with c-Src reintroduced (SYF-Src) were also obtained from the American Type Culture Collection. A 10 mmol/L stock of SKI-606 (Wyeth) in DMSO was diluted to the desired concentrations in culture medium before treatment. When exceeding 48 h treatment periods, redosing was scheduled every 2 d. The DMSO control was used at 0.01% or 0.0025% to correspond to the highest SKI-606 concentration used for each experiment.

Migration Assay and Video Time-Lapse Microscopy

Uniform "wounds" were made using a pipette tip on confluent monolayers of cells grown in 24-well plates or T-25 flasks (for video time-lapse microscopy), followed by immediate addition of the vehicle control (0.01% DMSO) or 0.01, 0.03, 0.1, 0.3, and 1 μ mol/L of SKI-606 as indicated. Cells were allowed to migrate into the denuded area for 48 h, then fixed and stained with a Coomassie blue solution (20). Photomicrographs were acquired with a 4 \times objective under brightfield illumination using a charge-coupled device camera-mounted Olympus IX81 Inverted microscope, and analyzed with Image-Pro Plus software (Media Cybernetics). For video time-lapse microscopy, flasks were gassed with 5% CO₂ and placed at 37°C for immediate imaging using 4 \times or 10 \times objectives from identically equipped Nikon TS100 Phase microscopes (Nikon) coupled to Sanyo video charge-coupled device cameras (Sanyo) and digitized at 640 \times 480 pixels with a Matrox frame grabber board (Matrox). Photomicrographs were captured every 2 min for each flask simultaneously for a total of 50 h. ImageJ version 1.36b (NIH) was used to process the images and the speed of migration was assessed using Image-Pro Plus software.

Invasion Assay

Twenty-four-well cell invasion chambers (Becton Dickinson) were used in accordance with the supplier's instructions. Cells (human and mouse) were suspended in 500 μ L serum-free medium and treated with SKI-606 (250 nmol/L or 1 μ mol/L) or the vehicle control DMSO (0.0025% or 0.01%) and were loaded into each upper invasion chamber. Cells were allowed to invade toward a lower chamber containing 750 μ L of freshly collected conditioned medium from each respective cell line for 48 h

at 37°C in 5% CO₂. Noninvasive cells were removed with PBS from the upper chamber and the remaining invasive cells stained using the Diff-Quik Stain kit (Dade Behring). The percent reduction in the number of invaded cells in treated wells compared with vehicle control-treated wells is presented.

Proliferation Assay

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays were done as described by the supplier (Promega). Briefly, ~5,000 cells were seeded in each well of a 96-well plate and allowed to adhere before the addition of 0.01% DMSO or 0.1, 0.3, and 1 μ mol/L of SKI-606. After 2 to 6 d of incubation at 37°C in 5% CO₂, MTS reagent was added to each well for 30 min and absorbance measured at 490 nm.

Growth in Three-Dimensional Culture

Anchorage-independent growth was assessed by growing 1 \times 10⁵ cells in six-well plates in a 0.33% agarose solution (Sigma) containing culture medium supplemented with 10% fetal bovine serum on top of a feeder layer of the same medium containing 0.7% agarose (21). Both agarose layers were supplemented with 0.01% DMSO or 1 μ mol/L SKI-606. Photomicrographs were taken 6 to 10 d later under brightfield illumination. Growth on a three-dimensional reconstituted basement membrane was done as previously described (22). Briefly, cells were seeded on a 100% Matrigel (BD Biosciences) basal layer containing 1 μ mol/L SKI-606 or 0.01% DMSO. A top layer of 4% Matrigel diluted in growth medium with the Src-inhibitor or vehicle control was used to overlay the cells. Clusters were allowed to grow for 4 to 6 d in a 37°C incubator before photomicrography.

Western Blot Analyses

Proteins were extracted using a buffer containing 50 mmol/L HEPES, 50 mmol/L NaCl, 1 mmol/L EGTA, 1% sodium deoxycholate, 10% glycerol, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na₃VO₄, 0.1 μ mol/L aprotinin, 1 μ mol/L leupeptin, and 1 μ mol/L antipain. Fifty micrograms of cell extract were resolved on a 8% polyacrylamide-SDS gel and transferred onto a polyvinylidene difluoride membrane (Millipore). The membranes were blocked with 4% ovalbumin for at least 1 h, followed by an overnight incubation with the following primary antibodies: phosphorylated Src, phosphorylated proline-rich tyrosine kinase 2 (Pyk2; BioSource International); Src (Upstate); phosphorylated FAK, FAK, phosphorylated p130^{Cas}, phosphorylated signal transducer and activator of transcription 3 (Stat3), Stat3, β -catenin, phosphorylated Akt, and poly(ADP-ribose) polymerase (Cell Signaling). Alexa Fluor 680-conjugated secondary antibodies were from Molecular Probes and IRDye 800 was from Li-Cor. Blots were analyzed using the Odyssey Infrared Imaging System (Li-Cor).

Immunofluorescence Microscopy

Cells were grown on a glass coverslip (1 \times 10⁵/mL) and treated with 0.01% DMSO or 1 μ mol/L SKI-606 for 48 h before fixation in 2% paraformaldehyde and 10 min permeabilization in PBS containing 0.5% Triton X-100

(PBST) at 4°C. Following three 20 min washes in PBST, cells were blocked with 0.1% bovine serum albumin and 10% goat serum for 1 h at room temperature. Cells were incubated overnight at 4°C with 1:200 anti-FAK pY576/577, 1:200 anti-FAK, 1:200 anti- β -catenin antibodies, or 1:200 anti-E-cadherin antibodies. Coverslips were washed thrice in PBST followed by incubation with the appropriate secondary antibody for 1 h at room temperature and staining with 4',6-diamidino-2-phenylindole (0.5 ng/mL in PBS) for 15 min. Fluorescent staining was examined using a Nikon TE2000-U inverted microscope equipped with a charge-coupled device camera and photomicrographs were taken with a 40 \times objective and Spot Software (Diagnostic Instruments).

Statistical Analysis and Reproducibility

Means and SEs are shown for all data sets. For the statistical analysis of invasion, we compared DMSO-treated cells versus SKI-606-treated cells with respect to reduction of objects invaded and used a one-sided, one-sample *t* test with a hypothesized value of 0% invasion reduction representing untreated cells. We also used the Wilcoxon

rank sum test as the method for comparing means in experiments where outliers violated assumptions of the traditional *t* test. All experiments were repeated at least in triplicate with similar results.

Results

SKI-606 Is a Potent Inhibitor of Breast Cancer Cell Spreading and Migration

Considerable evidence points to the importance of c-Src in regulating the dynamics of cell motility and adhesion (2–4). We therefore examined the effects of a 48 h treatment with SKI-606 on cell morphology and migration in different human cancer cell lines derived from breast cancer patients. Effects on cell morphology were observed at a concentration of 1 μ mol/L SKI-606 for all cell lines examined (representative morphologies are shown in Fig. 1A), and morphologic changes were apparent at concentrations as low as 0.25 μ mol/L (data not shown). SKI-606 caused the cells to adhere to each other, forming dense clusters as compared with vehicle control (DMSO)-treated cells, which showed spreading over larger areas.

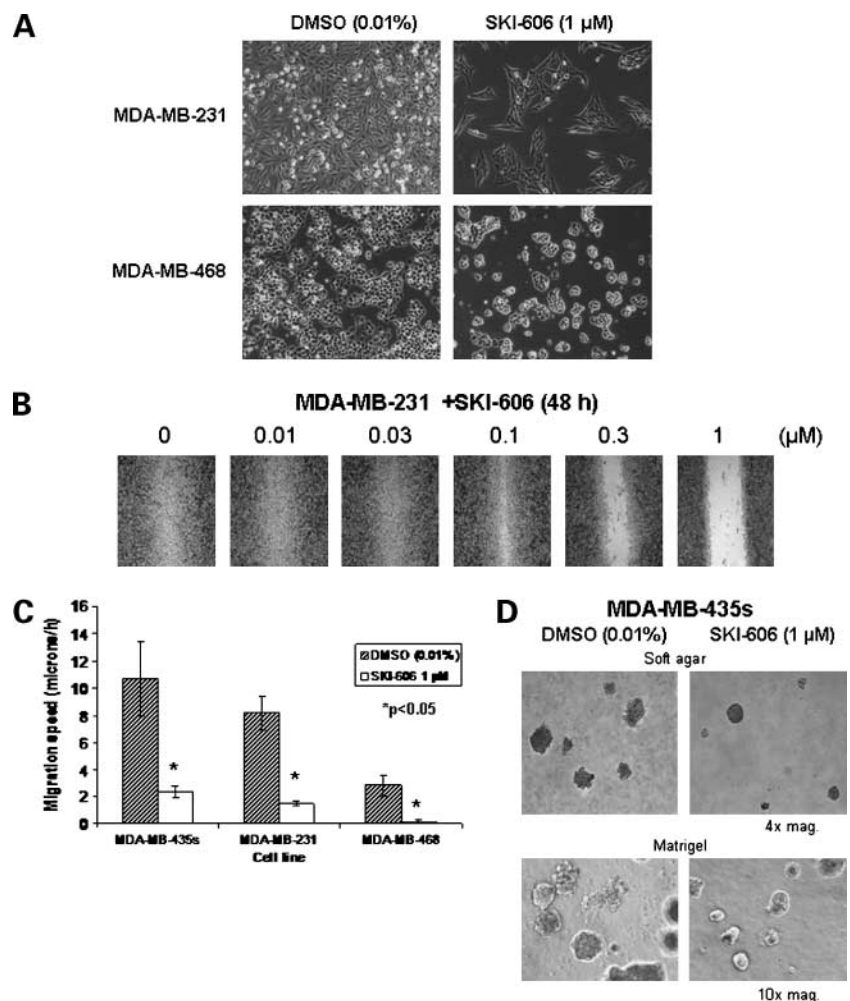


Figure 1. SKI-606 induces cell aggregation and decreases cell motility. **A**, MDA-MB-231 and MDA-MB-468 cell lines were cultured in the presence of 1 μ mol/L SKI-606 or 0.01% DMSO control for 96 h. Photomicrographs were taken under brightfield illumination ($\times 4$ magnification). **B**, wound assay done on the MDA-MB-231 cell line treated with 0, 0.01, 0.03, 0.1, 0.3, and 1 μ mol/L of SKI-606 and allowed to heal for 48 h before fixation and image capture (brightfield illumination; $\times 4$ magnification). DMSO (0.01%) was present in each assay well. **C**, summary of migration speeds assessed via video time-lapse microscopy. Breast cancer cell line migration was monitored in a denuded area of a confluent cell monolayer. Columns, mean speed value of three independent experiments; bars, SE. *, *P* < 0.05 (Wilcoxon test). **D**, MDA-MB-435s human breast cancer cells were grown in soft agar or Matrigel in the presence of 0.01% DMSO or 1 μ mol/L SKI-606 for 4 to 6 d and photomicrographs were taken under brightfield illumination ($\times 4$ and $\times 10$ magnifications).

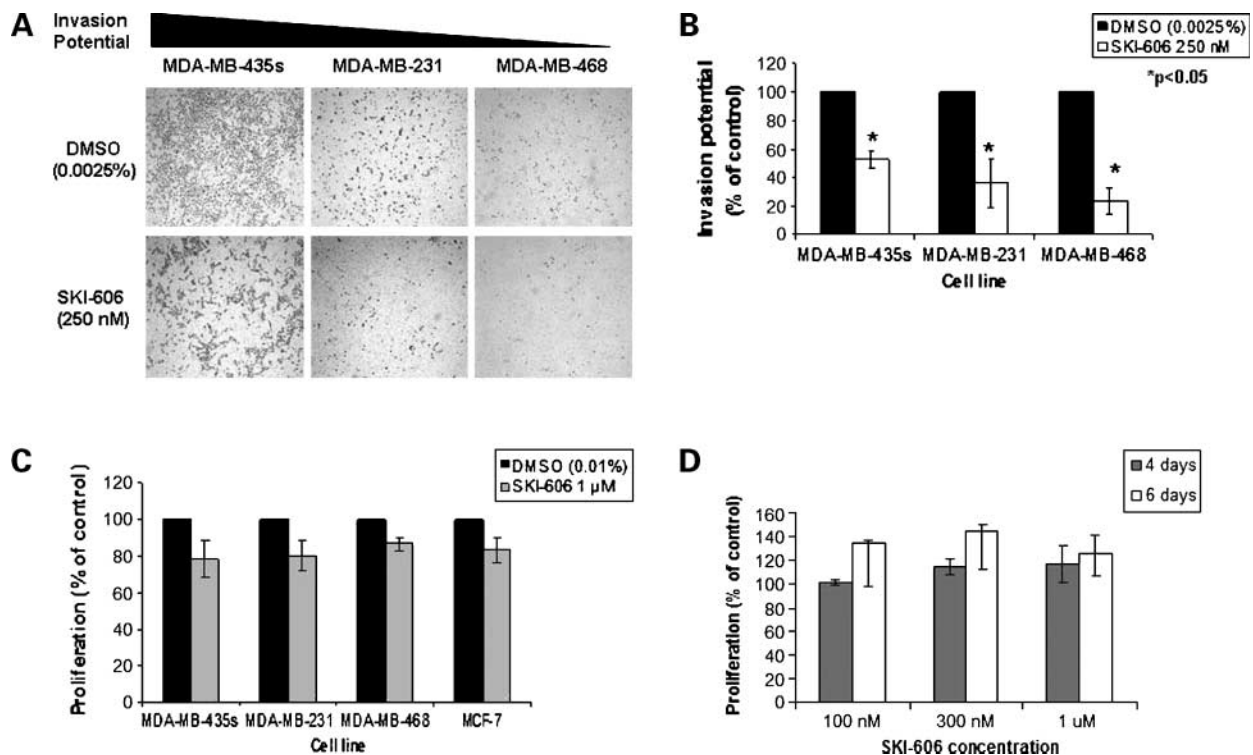


Figure 2. SKI-606 inhibits cell invasion but not proliferation. **A**, cells were seeded in an invasion chamber in the presence of 0.0025% DMSO vehicle control or 250 nmol/L SKI-606 and allowed to invade the chamber toward cell-specific conditioned medium for 48 h. Photomicrographs of stained invasive cells were taken under brightfield illumination ($\times 4$ magnification). **B**, invasion potential of breast cancer cell lines treated with SKI-606. Columns, mean percentage of invasive cells compared with the 0.0025% DMSO vehicle control – treated cells from three independent experiments; bars, SE. *, $P < 0.05$, versus a 0% invasion reduction (one-sided t test). **C**, breast cancer patient – derived cell lines were assessed for proliferation after treatment with 0.01% DMSO or 1 $\mu\text{mol/L}$ SKI-606 for 48 h using the MTS metabolic assay. Columns, mean percent reduction in absorbance values of cells treated with SKI-606 relative to untreated cells from three independent experiments; bars, SE. No statistically significant differences were observed using the Wilcoxon test. **D**, MDA-MB-231 cells were assessed for proliferation after treatment with 0.01% DMSO or 0.1, 0.3, or 1 $\mu\text{mol/L}$ SKI-606 for 4 and 6 d using the MTS metabolic assay. Columns, mean percent reduction in absorbance values of cells treated with SKI-606 relative to untreated cells from three independent experiments; bars, SE.

We also examined the effects of SKI-606 on cell migration using a “wound healing” assay. Exposure to increasing concentrations of SKI-606 inhibited migration of breast cancer cell lines with IC_{50} values of 0.1 to 0.3 $\mu\text{mol/L}$ (Fig. 1B). Using video time-lapse microscopy, we were able to quantify significant ($P < 0.05$) inhibition of cell migration speed after treatment with 1 $\mu\text{mol/L}$ SKI-606 (Fig. 1C). Similar observations were made with cancer cells grown in soft agar or on a three-dimensional reconstituted basement membrane; SKI-606 caused the formation of condensed aggregates with few extruding cells compared with the DMSO vehicle control – treated cells (Fig. 1D).

SKI-606 Blocks Tumor Cell Invasion but not Proliferation or Survival

We examined the ability of breast cancer patient – derived cell lines to invade a Matrigel layer and cross a porous membrane as a measure of invasive potential. After a 48 h treatment with 1 $\mu\text{mol/L}$ SKI-606, all the invasion-competent cell lines were unable to cross the porous membrane (data not shown), whereas at concentrations as low as 0.25 $\mu\text{mol/L}$ SKI-606, we observed a significant decrease in invasion potential (Fig. 2A and B). These

observations indicate a similar IC_{50} for SKI-606 – mediated inhibition of tumor cell migration and invasion. Using video time-lapse microscopy, we did not observe any effects of SKI-606 on cell proliferation or apoptosis associated with SKI-606 treatment over a 50 h period.⁴ To confirm these observations, MTS assays were done on breast cancer cell lines displaying different levels of active c-Src (23) after exposure to increasing concentrations of SKI-606. There were no significant changes in cell proliferation or viability for the cell lines examined after a 48 h treatment at concentrations exceeding the IC_{50} for Src inhibition (Fig. 2C). Similar observations were made after treatment with up to 1 $\mu\text{mol/L}$ of SKI-606 for 4 or 6 days (Fig. 2D).

SKI-606 Inhibits the Invasive Properties of Src, Yes, and Fyn Null Cells with Reintroduced c-Src

Src, Yes, and Fyn knockout mouse embryo fibroblasts (SYF^{-/-}), previously characterized by Klinghoffer et al.

⁴ Supplemental video time-lapse microscopy data available at <http://www.cityofhope.org/Researchers/JoveRichard/video>.

(24), provide an excellent system to determine the specificity of Src kinase inhibitors. Similar to the effects of SKI-606 on cell migration and invasion, SYF^{-/-} cells are defective in both of these processes (Fig. 3), consistent with

an essential role of Src family kinases in cell migration and invasion. We postulated that SKI-606 would have minimal effects on SYF^{-/-} cells, whereas SYF^{-/-} cells with reintroduced c-Src (SYF-Src) would exhibit restored sensitivity to

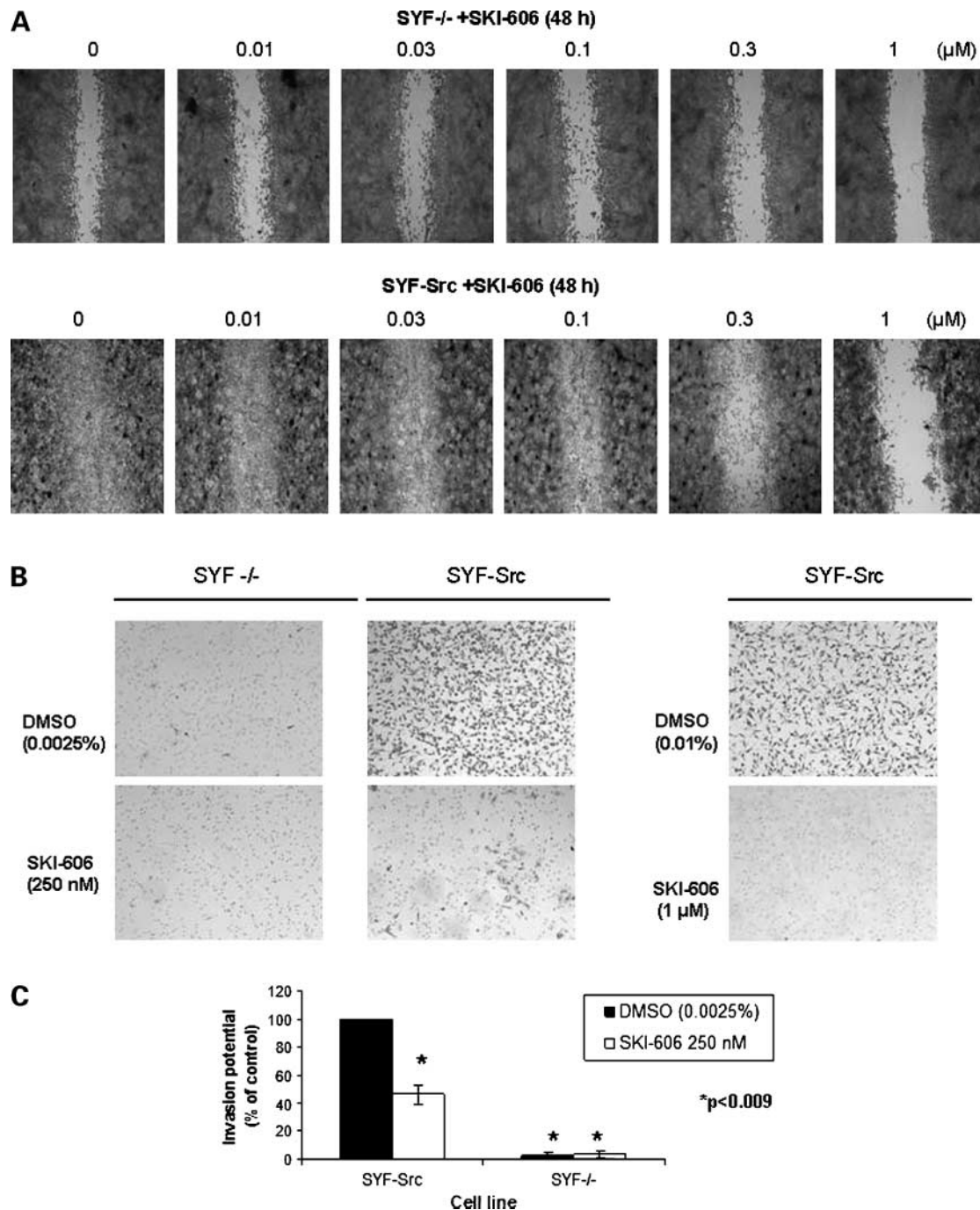


Figure 3. SKI-606 inhibits the invasive properties of SYF^{-/-} cells following reintroduction of c-Src. **A**, wound healing assay using SYF^{-/-} cells and SYF^{-/-} cells with reintroduced c-Src (SYF-Src), treated with 0.01% DMSO vehicle control or increasing concentrations of SKI-606 and allowed to migrate for 48 h. **B**, cells were seeded in serum-free medium in the top layer of an invasion chamber in the presence or absence of 0.01% DMSO, 250 nmol/L SKI-606, or 1 μmol/L SKI-606 and allowed to invade the chamber toward cell-specific conditioned medium for 48 h. Photomicrographs were taken under brightfield illumination (×4 magnification). **C**, invasion potential of SYF^{-/-} and SYF-Src cells treated with 0.0025% DMSO or 250 nmol/L SKI-606. Columns, mean percentage of invasive cells compared with DMSO vehicle control-treated SYF-Src cells from three independent experiments; bars, SE. *, $P < 0.009$, versus SYF-Src cells with 100% invasion potential (one-sided t test).

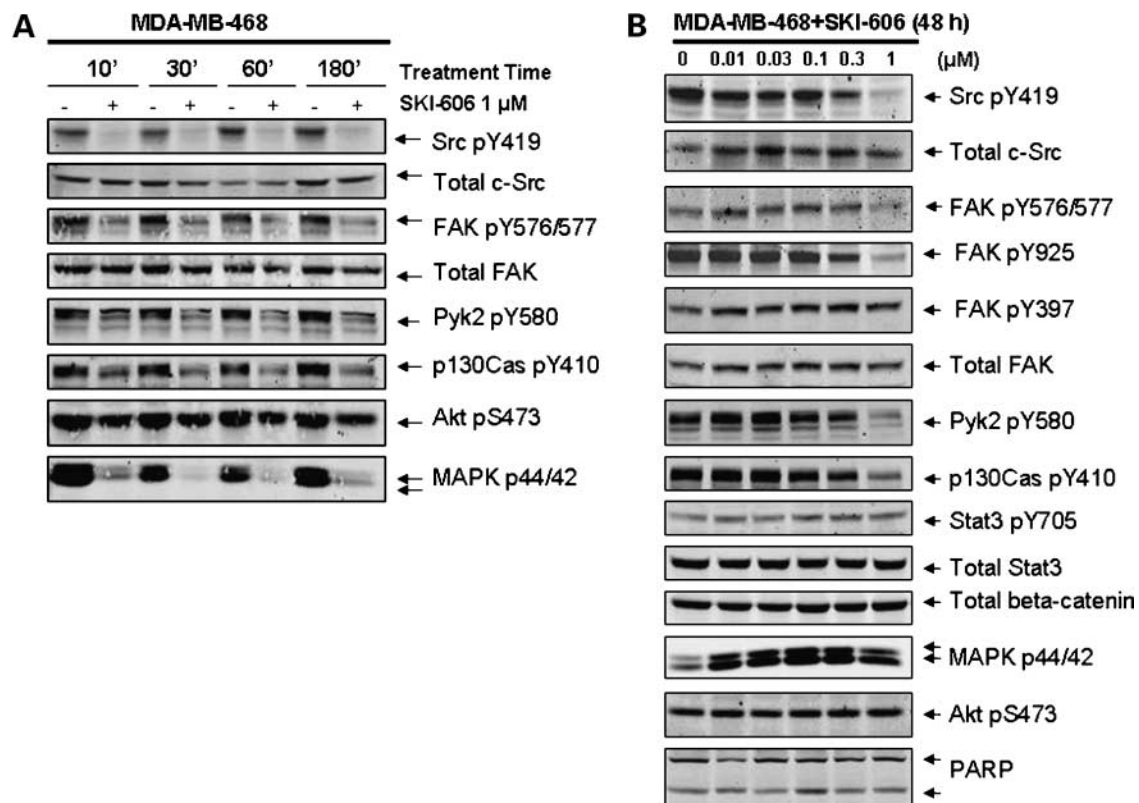


Figure 4. SKI-606 causes rapid and prolonged inhibition of Src/FAK/Pyk2/p130^{Cas} phosphorylation. Western blot analysis of MDA-MB-468 whole-cell extracts. Cells were treated with SKI-606 or 0.01% DMSO at the indicated concentrations for times up to 3 h (A) or 48 h (B) before extraction. Immunoblots were probed as indicated with antibodies to phospho-Src (pY419), phospho-FAK (pYpY576/577 or pY925), phospho-Pyk2 (pY580), phospho-p130^{Cas} (pY410), phospho-Stat3 (pY705), phospho-Akt (pS473), phospho-mitogen-activated protein kinase (MAPK) p44/42, β -catenin, or poly(ADP-ribose) polymerase (PARP). Blots were reprobed with total anti-Src, anti-FAK, or anti-Stat3 antibodies, as indicated.

SKI-606. We therefore carried out a wound healing assay on SYF^{-/-} and SYF-Src cells and compared the effects of increasing concentrations of SKI-606 on cell migration into the denuded area. After a 48 h treatment of SYF^{-/-} cells with SKI-606, only minor effects could be visualized at 1 μ mol/L SKI-606 compared with the DMSO vehicle control (Fig. 3A). By contrast, SYF-Src cells completely covered the denuded area after 48 hours, and this process was inhibited by 0.3 μ mol/L or higher concentrations of SKI-606 (Fig. 3A). When assessed for invasion potential over 48 hours, SYF^{-/-} cells were unable to cross Matrigel invasion chambers; however, SYF-c-Src cells were highly invasive unless treated with SKI-606 at concentrations of ≥ 0.25 μ mol/L (Fig. 3B and C). These findings provide genetic evidence that c-Src is required for inhibition of cell migration and invasion by SKI-606.

SKI-606 Inhibits Src, FAK, and p130^{Cas} Phosphorylation

To determine the effects of SKI-606 on signaling pathways within our human cancer cell lines, we investigated several phosphorylated downstream effectors of Src in multiple human cell lines including MDA-MB-468, MDA-MB-231, MDA-MB-435s, MDA-MB-453, and MCF-7. We observed a rapid (within 10 minutes; Fig. 4A) and

prolonged concentration-dependent inhibition (at 48 hours; Fig. 4B) in these cells of phosphorylated Tyr576, Tyr577, Tyr925 on FAK, Tyr580 on Pyk2, and Tyr410 on p130^{Cas}. This inhibition was coincident with the decline of Src autophosphorylation at Tyr419, which reflects Src kinase activity in cells, with an IC₅₀ of ~ 300 nmol/L. No significant changes were observed in phosphorylated Tyr397 on FAK (an autophosphorylation site; ref. 25), showing that FAK intrinsic kinase activity is not affected by SKI-606, whereas the Src-dependent FAK phosphorylation sites (Tyr576/577, Tyr925) are inhibited by SKI-606. No changes in total protein levels were observed for any of the signaling proteins examined despite the obvious changes in phosphorylation, and similar observations were made for all breast cancer patient-derived cell lines tested (data not shown).

We also examined other signaling pathways previously shown to be regulated by Src in different cellular contexts (Fig. 4). No changes were observed in the phosphorylation of Tyr705 in Stat3 and Ser473 in Akt at SKI-606 concentrations that decrease Src Tyr419 autophosphorylation, indicating that SKI-606 selectively inhibits the Src/FAK/Pyk2/p130^{Cas} pathway in these breast cancer cell lines. This lack of inhibition of phosphorylated Stat3 and Akt,

which are important in tumor cell survival, is consistent with our finding that SKI-606 does not induce apoptosis in breast cancer cells. Lack of poly(ADP-ribose) polymerase cleavage further supports the observation that apoptosis was not induced by SKI-606 treatment of these cells. In addition, although p44/p42 phosphorylation on Thr202/Tyr204 was inhibited at very early times (180 minutes; Fig. 4A) of SKI-606 treatment, by 48 hours the phosphorylation levels of p44/p42 were restored (Fig. 4B), consistent with the observed lack of inhibition of cell proliferation by SKI-606 under these conditions. Analysis of the same signaling pathways after treatment with SKI-606 for 6 days (with redosing every 2 days) revealed results similar to those found at 48 hours (data not shown), indicating that these signaling pathways stabilize after 48 hours despite the initial changes observed after immediate treatment with SKI-606.

SKI-606 Causes an Increase in Membrane-Localized β -Catenin and Stabilization of Cell-to-Cell Adhesions

Given our observations that SKI-606 causes cell motility defects and changes in FAK phosphorylation, we examined the localization of Src effectors found in focal adhesions in SKI-606-treated and untreated cells. Immunofluorescence microscopy revealed the disappearance of FAK pY576/pY577 after SKI-606 treatment, supporting our findings by Western blot analysis, whereas total FAK protein staining was unchanged in the presence of SKI-606 despite the lack of migrating leading fronts (lamellipodia and/or filipodia; Fig. 5A). These findings agree with the suggested role of c-Src in the dynamic turnover of focal adhesions rather than in their assembly (26). Interestingly, pY705-Stat3 failed to localize to focal adhesions despite total levels of the activated protein remaining unchanged. These observations agree with the findings of Silver et al. (27) who suggest that Src family members are required for normal localization of Stat3. Significantly, cell aggregation after treatment with SKI-606 was accompanied by an increase in membrane-localized β -catenin (Fig. 5A and B) although total levels of β -catenin remained unchanged (Fig. 4B). These findings suggest the possibility that SKI-606 increases cell-to-cell adhesion via β -catenin-mediated stabilization of cell-surface adhesion molecules.

Discussion

Src kinases are transducers of signals activated by many different classes of cell-surface receptors; they interact with a large number of substrates and they mediate a wide array of biological events. Therefore, predicting the outcome of interfering with these key effectors is not straightforward. What is clear, however, through an increasing number of studies using a new generation of more selective small-molecule Src inhibitors, is that targeting Src family kinase activity results in potent antineoplastic effects in a wide variety of different tumor cell types (14, 16, 28, 29). Whereas differences arise in the biological effects of these compounds in terms of cell proliferation, survival, adhesion, and morphology, likely caused by off-

target effects, inhibition of cell migration and invasion is consistently a recurring response (13, 15, 28, 30, 31).

Our present findings using the Src inhibitor SKI-606 on human cancer cell lines obtained from breast cancer patients show reduced cell migration and invasion. In addition, we show that these effects are accompanied by an increase in cell-to-cell adhesion. These responses occur at concentrations corresponding to detectable inhibition of Src autophosphorylation on Tyr419, suggesting that c-Src

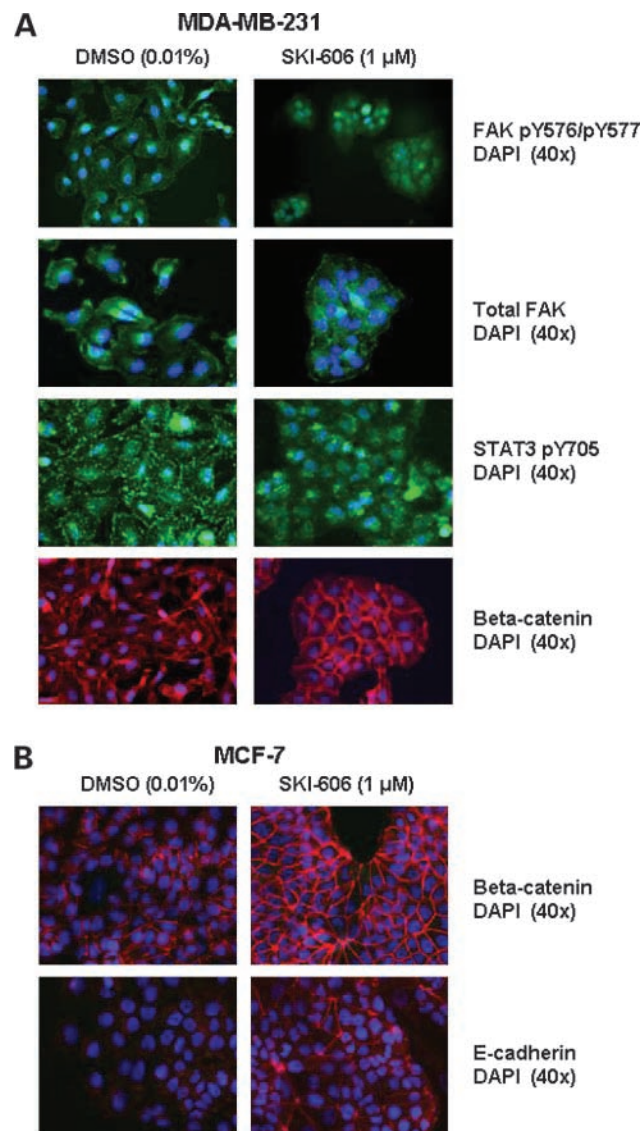


Figure 5. Immunofluorescence analysis of FAK and β -catenin in cells treated with SKI-606. **A**, MDA-MB-231 cells were treated with 0.01% DMSO or 1 μ mol/L SKI-606 for 48 h, then fixed, probed with antibodies to phospho-FAK (pY576/pY577), FAK, phospho-Stat3 (pY705), or β -catenin as indicated, and stained with 4',6-diamidino-2-phenylindole (DAPI). Images were captured under fluorescence at $\times 40$ magnification. **B**, MCF-7 cells were treated with 0.01% DMSO or 1 μ mol/L SKI-606 for 48 h, then fixed, probed with antibodies to β -catenin or E-cadherin, and stained with 4',6-diamidino-2-phenylindole. Images were captured under fluorescence at $\times 40$ magnification.

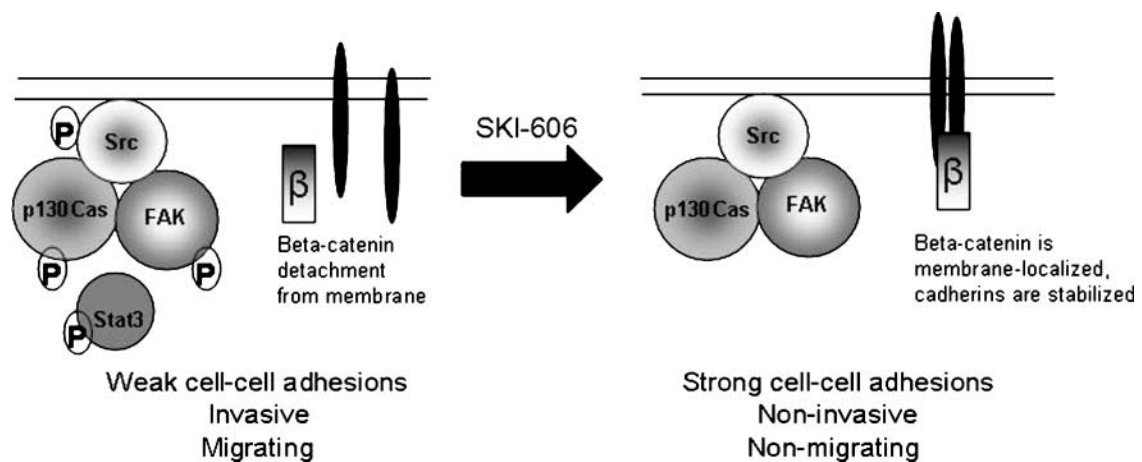


Figure 6. Model for mechanism of action of SKI-606 in human breast cancer cells. SKI-606 inhibits Src kinase activity and thereby disrupts phosphorylation of the Src/FAK/p130^{Cas} multiprotein complex. SKI-606 also increases β -catenin localization to the membrane and leads to loss of phosphorylated Stat3 from focal adhesion sites. The biological consequences of SKI-606 treatment are enhanced cell-to-cell adhesion, as well as reduced cell migration and invasion.

plays a key role in these events. The important role of c-Src signaling in mediating the response to SKI-606 is further shown by our experiments using SYF^{-/-} and SYF-Src cells. SKI-606 had minimal effects on SYF^{-/-} cells, which, when left untreated, migrated slowly and were unable to cross a Matrigel invasion chamber over 48 h. However, the same cells with c-Src reintroduced (SYF-c-Src) were highly migratory and invasive, unless treated with SKI-606, which inhibited cell migration and invasion. The ability of c-Src to have such effects on cell migration and invasion, and for these effects to be blocked by SKI-606 at concentrations correlating with inhibition of Src kinase activity, provides compelling evidence that SKI-606 mediates its biological responses through inhibition of c-Src kinase. In addition, our results point toward the enhanced specificity of SKI-606 in targeting the Src kinase because SYF^{-/-} cells seemed to be mostly unaffected by the addition of the small-molecule inhibitor.

Our data show that decreased cell motility and invasion are not associated with significant changes in cell proliferation or apoptosis. Thus, in the cancer cell lines studied herein, the signaling pathways responsible for cell proliferation and survival do not rely heavily on Src kinase activity. In particular, we show that phosphorylated Stat3, Akt, and mitogen-activated protein kinase levels are not decreased after extended exposure to SKI-606, consistent with the lack of effect on cell proliferation and survival. Furthermore, these signaling pathways can recover from Src kinase inhibition over time, as observed for mitogen-activated protein kinase phosphorylation at 3 hours versus 48 hours posttreatment. In striking contrast, low levels of phosphorylated Src, FAK, and p130^{Cas} are observed at 10 minutes and remain low even 6 days posttreatment, consistent with inhibition of migration and invasion. However, under conditions of reduced serum levels in the culture media, prolonged treatment with SKI-606 has

been found to inhibit growth of some breast cancer cells (32). It is possible that the response of tumor cells to SKI-606 depends on the particular signaling circuitry and the ability of the cell to overcome Src inhibition by up-regulating other pathways involved in growth and survival. We also found that cancer cells grown on three-dimensional reconstituted basement membranes or in soft agar and treated with SKI-606 formed condensed aggregates with few extending projections, suggesting that our experiments conducted in two-dimensional monolayer cultures are indicative of potential three-dimensional *in vivo* responses.

FAK is phosphorylated by Src on a number of tyrosine residues and, similar to Src, is also associated with malignant progression of breast cancer (33, 34). Given that Src-mediated activation of FAK negatively regulates cell-to-cell adhesion (35), the decrease in FAK phosphorylation on Src-dependent sites could at least partially account for the cell aggregation phenotype we observed after SKI-606 treatment. In addition, decreased phosphorylation of FAK on Tyr925 following SKI-606 treatment is correlated with the observed reduction in motility. These results agree with earlier findings by Brunton et al. (36), indicating that the Src kinase-dependent phosphorylation of Tyr925 in FAK is important in controlling the extension and retraction of cell protrusions or adhesion turnover. p130^{Cas}, another substrate of c-Src with decreased phosphorylation following SKI-606 treatment, is also involved in cell spreading, focal adhesion formation, motility, and invasion, and its high expression is associated with poor prognosis in breast cancer patients (37). As a scaffold protein, p130^{Cas} associates with Src, FAK, Pyk2, and other signaling molecules in multiprotein complexes. Following treatment with SKI-606, we observed a decrease in phosphorylated Pyk2 at Tyr580, a Src-specific phosphorylation site. Pyk2, also known as RAFTK/CADTK/FAK2/CAK β , was previously

shown to have an important role in transducing chemotactic signals in breast cancer cell lines (38) and mediating cell-cell adhesion by controlling β -catenin phosphorylation (39).

Earlier studies using colorectal cells suggest that SKI-606 causes cell aggregation (19). Interference with Src-mediated tyrosine phosphorylation of β -catenin in this case could be regulating a switch between the adhesive and transcriptional functions of β -catenin, thus promoting cell-to-cell adhesion and stabilizing E-cadherin proteins on the cell surface (29). Following treatment with SKI-606, our breast cancer patient-derived cell lines also display tighter aggregates, associated with higher levels of membrane-localized β -catenin. However, the cell-surface receptors involved in SKI-606-mediated cell adhesion differ across cell lines and may be of secondary importance to this effect because both E-cadherin-positive and E-cadherin-negative cells form tight aggregates and exhibit reduced migration and invasion. Finally, the observed lack of Stat3 pY705 localized to focal adhesions, despite total activated levels of Stat3 pY705 remaining unchanged, points to the potential involvement of Stat3 in the invasive phenotype of human cancer cells in addition to their survival. However, the role of Stat3 in focal adhesions remains to be determined.

In sum, our studies showing decreased cell motility and invasion, as well as increased cell-cell adhesion, following SKI-606 treatment suggest that SKI-606 has potential for the treatment of breast cancer and possibly other tumor sites. SKI-606 interferes with key cellular mechanisms and signaling pathways relied on extensively by cancer cells for invading and metastasizing, whereas cell proliferation and survival are not inhibited (summarized in Fig. 6). Thus, Src kinase inhibitors such as SKI-606 may act independently of cytotoxic agents and instead enhance long-term survival of breast cancer patients by preventing tumor cell invasion and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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