

Dasatinib (BMS-354825) Selectively Induces Apoptosis in Lung Cancer Cells Dependent on Epidermal Growth Factor Receptor Signaling for Survival

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Abstract

Mutations of the epidermal growth factor receptor (EGFR) selectively activate Akt and signal transducer and activator of transcription (STAT) pathways that are important in lung cancer cell survival. Src family kinases can cooperate with receptor tyrosine kinases to signal through downstream molecules, such as phosphatidylinositol 3-kinase/PTEN/Akt and STATs. Based on the importance of EGFR signaling in lung cancer, the known cooperation between EGFR and Src proteins, and evidence of elevated Src activity in human lung cancers, we evaluated the effectiveness of a novel orally bioavailable Src inhibitor dasatinib (BMS-324825) in lung cancer cell lines with defined EGFR status. Here, we show that cell fate (death versus growth arrest) in lung cancer cells exposed to dasatinib is dependent on EGFR status. In cells with EGFR mutation that are dependent on EGFR for survival, dasatinib reduces cell viability through the induction of apoptosis while having minimal apoptotic effect on cell lines with wild-type (WT) EGFR. The induction of apoptosis in these EGFR-mutant cell lines corresponds to down-regulation of activated Akt and STAT3 survival proteins. In cell lines with WT or resistant EGFR mutation that are not sensitive to EGFR inhibition, dasatinib induces a G₁ cell cycle arrest with associated changes in cyclin D and p27 proteins, inhibits activated FAK, and prevents tumor cell invasion. Our results show that dasatinib could be effective therapy for patients with lung cancers through disruption of cell growth, survival, and tumor invasion. Our results suggest EGFR status is important in deciding cell fate in response to dasatinib. (Cancer Res 2006; 66(11): 5542-8)

Introduction

Activating mutations in the tyrosine kinase domain of the epidermal growth factor (EGF) receptor (EGFR) selectively activate Akt and signal transducer and activator of transcription (STAT) pathways important in lung cancer cell survival and can predict sensitivity to small-molecule inhibitors of EGFR, such as gefitinib and erlotinib (1–4). Cell lines harboring activated EGFR molecules are dependent on EGFR for survival because inhibition of EGFR

results in apoptosis (4, 5). It has become increasingly clear that Src family kinases can link signals originating from growth factor, integrin, and cytokine receptors on the surface of cells to their downstream effector signaling cascades (6). c-Src cooperates with EGFR and ErbB2 and can be necessary for transformation by EGFR (7, 8). In addition, c-Src directly modulates EGFR function through phosphorylation of tyrosine residues on EGFR that allows for coupling to downstream signaling events (7). Src family kinases can cooperate with receptor tyrosine kinases to signal through downstream molecules, such as phosphatidylinositol 3-kinase (PI3K)/PTEN/Akt and STATs (6, 7, 9). For these reasons, we surmised that cell lines harboring activating EGFR mutations may show increased sensitivity to Src inhibitors. We evaluated the antitumor efficacy of a novel orally bioavailable Src inhibitor dasatinib (BMS-354825) in cell lines with defined EGFR status, including wild-type (WT) and mutant EGFR sensitive to gefitinib (10). In addition to Src proteins, dasatinib can potentially interact with other important tyrosine kinase proteins involved in tumor cell growth and survival and these interactions could enhance its antitumor activity (11).

Materials and Methods

Cell lines and cell culture. Human lung cancer cell lines were maintained in RPMI 1640 plus 5% bovine calf serum (BCS). H3255 cells were provided by Dr. Pasi Janne (Dana-Farber, Boston, MA) and grown in ACL-4 medium (12). HCC827 cells were provided by Dr. Jon Kurie (M.D. Anderson, Houston, TX). PC9 cells were provided by Dr. Matthew Lazzara (Massachusetts Institute of Technology, Boston, MA). Stock solutions of gefitinib and dasatinib in 100% DMSO were diluted directly into the medium to indicated concentrations. Gefitinib was provided by AstraZeneca (Wilmington, DE) and dasatinib by Bristol-Myers Squibb Oncology (Princeton, NJ). For cell transfection experiments, 2×10^6 HEK293 cells in a 6-cm dish maintained in DMEM/10% BCS were transfected with 1 μ g plasmid DNA for 3 hours using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) and then allowed to go 24 hours before being treated with inhibitors.

Cytotoxicity and apoptosis assays. Cytotoxicity assays [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were done according to the manufacturer's recommendations (Roche, Indianapolis, IN). Cells (5×10^3) in medium with 5% BCS were placed into single wells in a 96-well plate and exposed to indicated agents, and viability was assessed after 72 hours. Data presented represent three separate experiments with eight data points per concentration per experiment. Apoptosis [PharMingen (San Diego, CA) Apo-BrdU kit] and cell cycle changes (propidium iodide staining and flow cytometry) were assayed as before (13). Data are expressed as mean \pm SD.

Invasion assays. Boyden chambers (8 μ m pores; Costar, Fisher, Corning, NY) were loaded with 10 μ g growth factor-depleted/reduced Matrigel (BD Biosciences, San Diego, CA) and air dried overnight. Cells (100,000) in medium plus 0.1% bovine serum albumin were loaded onto the top chamber, whereas complete medium was added to lower chamber, and

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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chambers were loaded in duplicate and placed back into the incubator. After 22 hours, the filters were removed, wiped with a cotton swab to remove Matrigel and noninvading cells, and stained with DiffQuick. Five fields were counted for invading cells per filter.

Protein expression analysis. Cell lysates were prepared using radioimmunoprecipitation assay buffer [10 mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 20 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 2 mmol/L Na_3VO_4 , 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 60 $\mu\text{g}/\text{mL}$ aprotinin, 10 $\mu\text{g}/\text{mL}$ leupeptin, 1 $\mu\text{g}/\text{mL}$ pepstatin], normalized for total protein content (50 μg), and subjected to SDS-PAGE. Primary antibodies used in these studies consisted of phosphotyrosine (pTyr)⁸⁴⁵ EGFR, pTyr¹⁰⁶⁸

EGFR (pEGFR), total EGFR, pSer⁴⁷³ Akt (pAkt), total Akt, pTyr⁷⁰⁵ STAT3 (pSTAT3), total STAT3, pTyr⁴¹⁶ Src (pSrc) family, c-Src, pTyr⁸⁶¹ FAK (pFAK), total FAK, p42/44 extracellular signal-regulated kinase (ERK), total ERK, and cleaved poly(ADP-ribose) polymerase (PARP; Cell Signaling, Danvers, MA). Cyclins D1 and D3 and p27 antibodies have been described previously (14).

Results

To assess dasatinib sensitivity of non-small cell lung cancer (NSCLC) cells harboring distinct EGFR mutations, cell lines containing the L858R mutation (H3255), L858R + T790M (H1975), and deletion mutation (HCC827, PC9, and H1650) along with cell

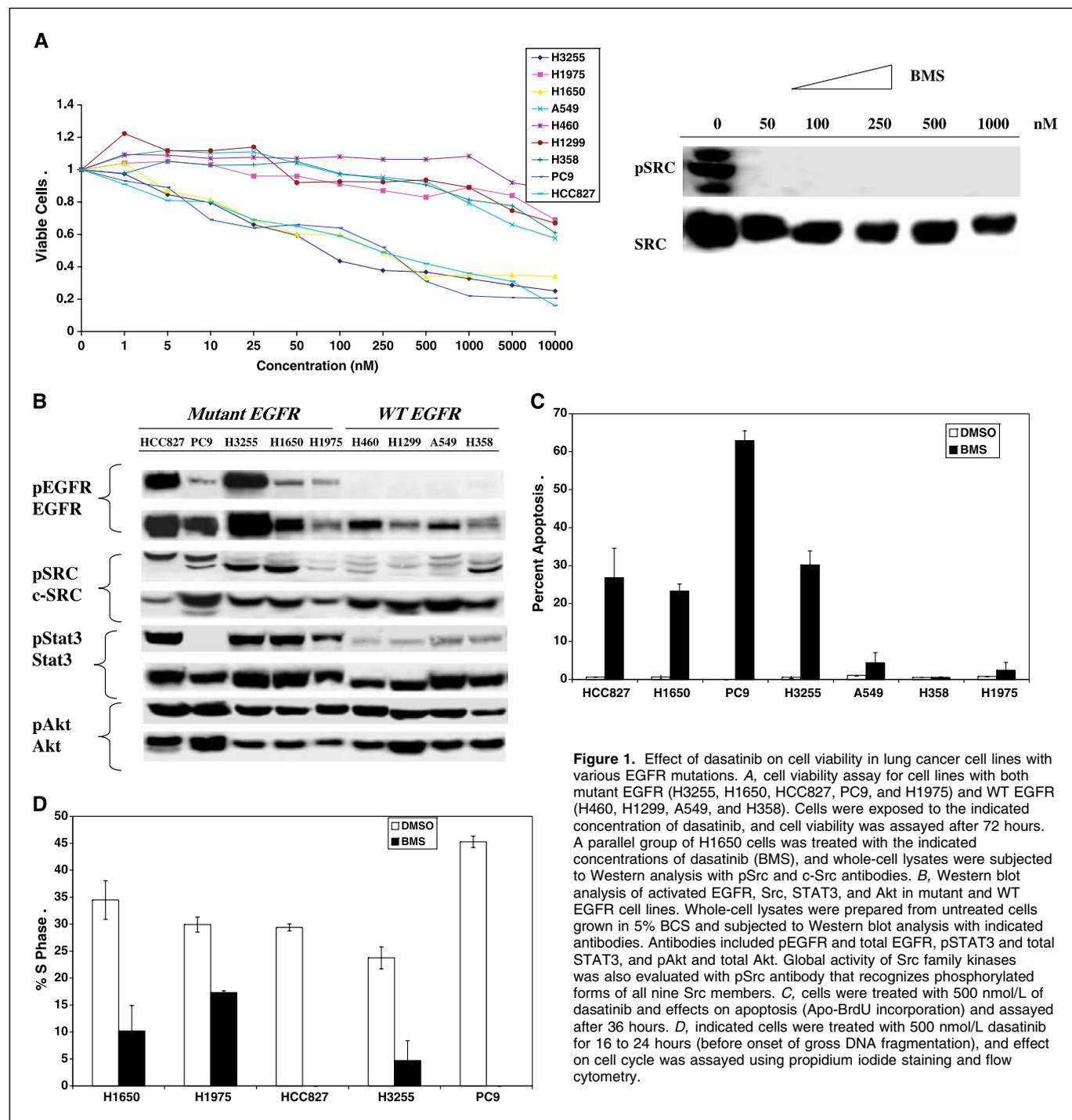


Figure 1. Effect of dasatinib on cell viability in lung cancer cell lines with various EGFR mutations. **A**, cell viability assay for cell lines with both mutant EGFR (H3255, H1650, HCC827, PC9, and H1975) and WT EGFR (H460, H1299, A549, and H358). Cells were exposed to the indicated concentration of dasatinib, and cell viability was assayed after 72 hours. A parallel group of H1650 cells was treated with the indicated concentrations of dasatinib (BMS), and whole-cell lysates were subjected to Western analysis with pSrc and c-Src antibodies. **B**, Western blot analysis of activated EGFR, Src, STAT3, and Akt in mutant and WT EGFR cell lines. Whole-cell lysates were prepared from untreated cells grown in 5% BCS and subjected to Western blot analysis with indicated antibodies. Antibodies included pEGFR and total EGFR, pSTAT3 and total STAT3, and pAkt and total Akt. Global activity of Src family kinases was also evaluated with pSrc antibody that recognizes phosphorylated forms of all nine Src members. **C**, cells were treated with 500 nmol/L of dasatinib and effects on apoptosis (Apo-BrdU incorporation) and assayed after 36 hours. **D**, indicated cells were treated with 500 nmol/L dasatinib for 16 to 24 hours (before onset of gross DNA fragmentation), and effect on cell cycle was assayed using propidium iodide staining and flow cytometry.

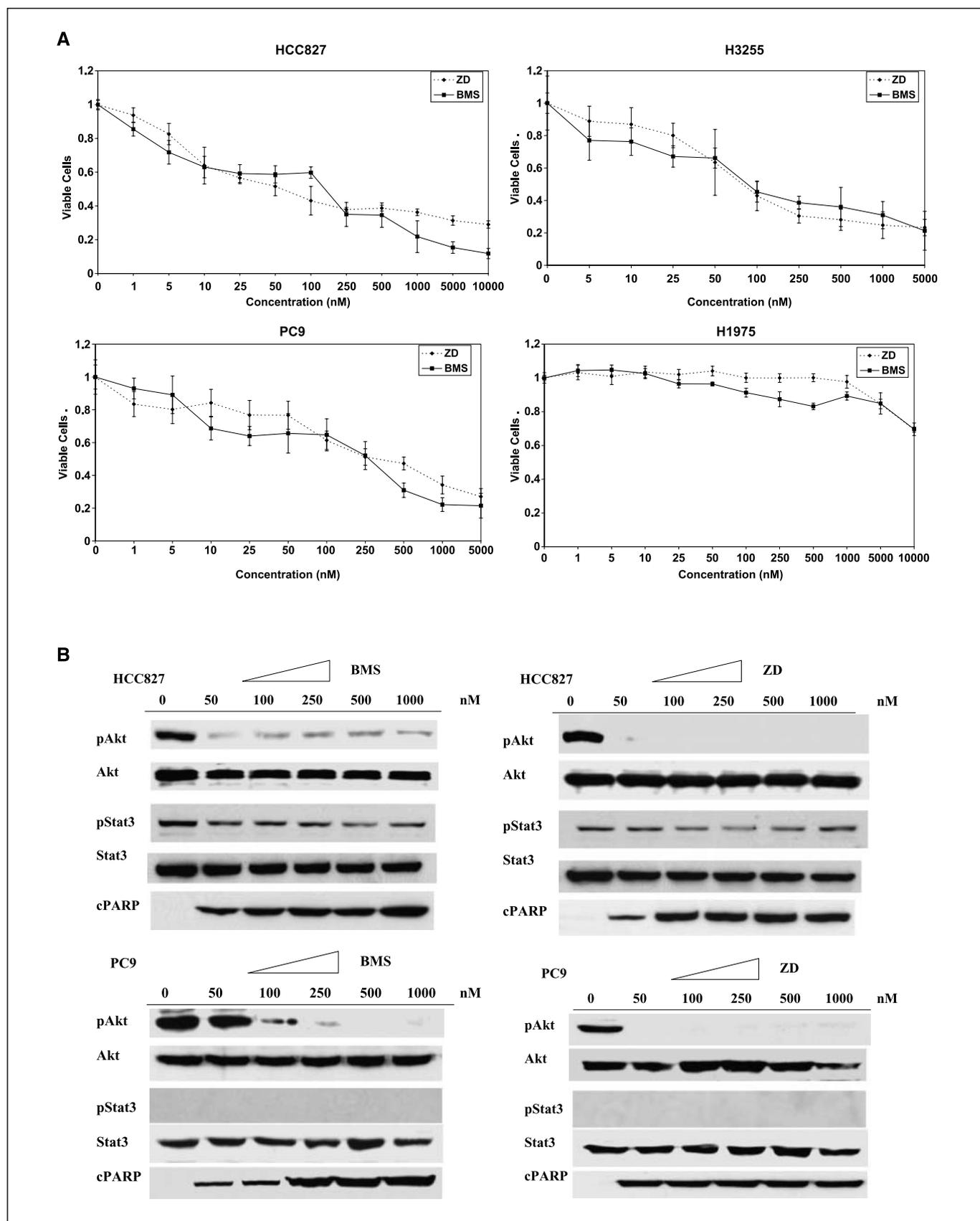


Figure 2. Dasatinib induces apoptosis in EGFR-mutant NSCLC through down-regulation of Akt and STAT3. *A*, indicated cells were exposed to increasing concentrations of gefitinib or dasatinib, and cell viability was assessed at 72 hours. *B*, mutant EGFR cell lines were exposed to indicated concentrations of either gefitinib or dasatinib, and total proteins were collected after 24 hours. Membranes were blotted with indicated antibodies.

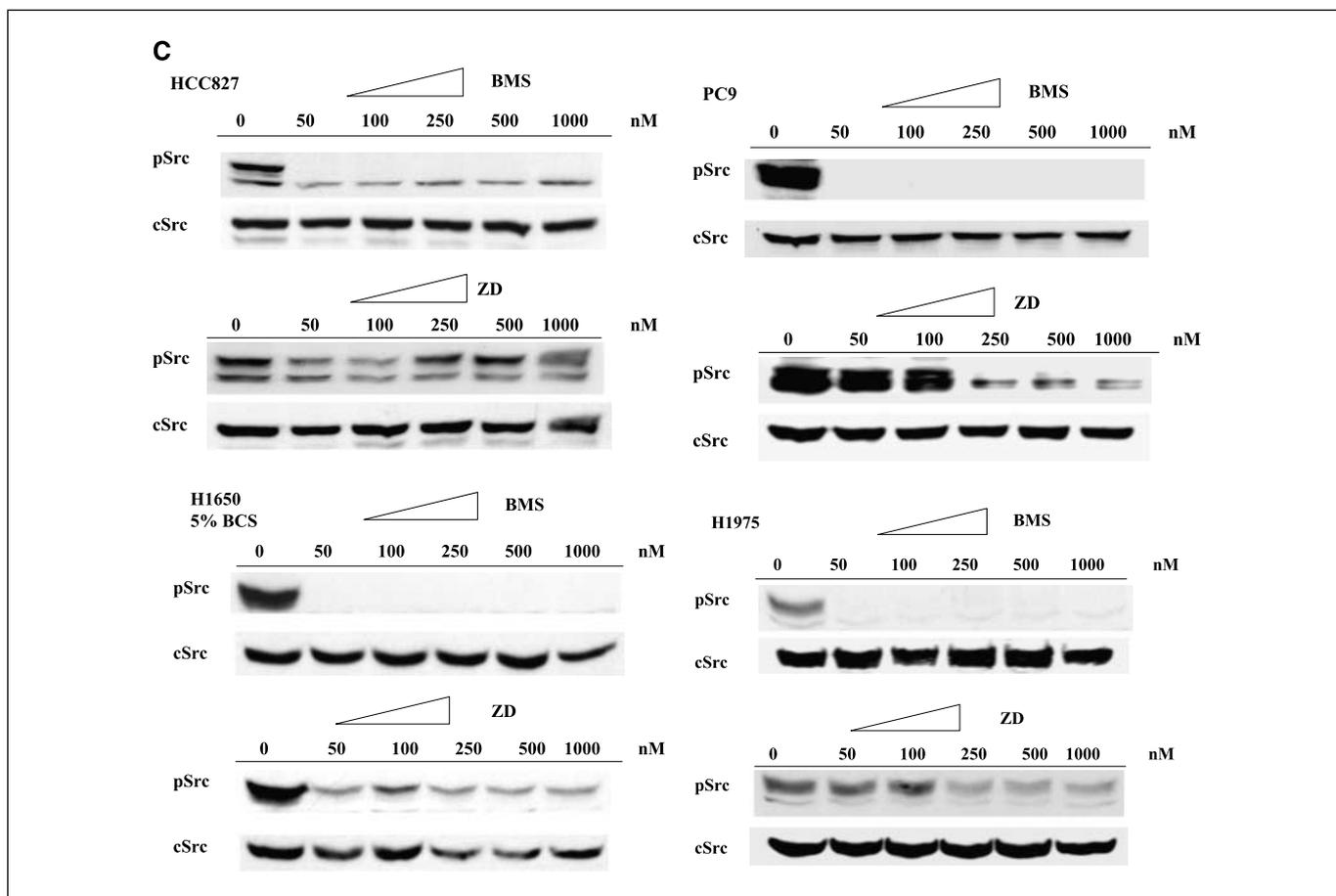


Figure 2 Continued. C, indicated cells were exposed to increasing concentrations of either dasatinib or gefitinib for 24 hours, and whole-cell lysates were evaluated for pSrc and total c-Src using Western analysis. H1650 cells were grown in either 5% BCS or 0.5% BCS plus supplemental 100 ng/mL EGF. ZD, gefitinib.

lines with WT EGFR (H460, H358, H1299, and A549) were exposed to increasing concentrations of dasatinib and cell viability was assessed. As shown in Fig. 1A, mutant EGFR cells are sensitive to dasatinib with an approximate IC_{50} of 100 to 250 nmol/L, whereas WT EGFR and H1975 cells are resistant to dasatinib (IC_{50} >10 μ mol/L). Dasatinib completely inhibits autophosphorylation of Tyr⁴¹⁶ on Src family members at a concentration of 50 nmol/L in H1650 cells (lower concentrations are not evaluated). An untreated group of parallel cells was evaluated for activated EGFR, Src family kinases, STAT3, and Akt. An antibody reflecting autophosphorylation of pTyr⁴¹⁶ on Src family proteins recognizes several distinct bands in the 56- to 60-kDa region with the suggestion of more expression in cells sensitive to dasatinib. We found that cell lines with mutant EGFR (H3255, H1650, PC9, HCC827, and H1975) have enhanced levels of pEGFR and pSTAT3 compared with WT EGFR cells (H460, H358, H1299, and A549), with PC9 being the exception because it has undetectable pSTAT3 expression (Fig. 1B). To assess how dasatinib affects cell viability, we assayed EGFR-mutant cell lines for changes in cell cycle and apoptosis. Dasatinib resulted in apoptosis in cells with EGFR mutants sensitive to gefitinib (H3255, H1650, HCC827, and PC9), whereas minimal apoptosis was observed in WT EGFR cells (A549 and H358) or in gefitinib-resistant H1975 cells (Fig. 1C). In addition to undergoing apoptosis, dasatinib inhibits DNA synthesis in cells with EGFR mutation, including H1975 (Fig. 1D).

We next directly compared the effect of dasatinib and gefitinib on cell viability in cells with gefitinib-sensitive or gefitinib-resistant EGFR mutation. Because cell growth conditions can affect sensitivity of cells to gefitinib, we repeated cell viability assays comparing dasatinib with gefitinib (15). In these cells, changes in cell viability as a function of concentration of inhibitor were similar to both gefitinib and dasatinib, with H1650 being the one exception because dasatinib inhibited cell viability more than gefitinib when grown in 5% BCS (Fig. 2A; Supplementary Fig. S2A). We next evaluated how dasatinib affects EGFR-mediated survival signaling through STAT3 and Akt. The choice of these molecules was based on their role in mutant EGFR-dependent survival signaling as well as being downstream targets for Src signaling (4, 5, 12, 16). Cells were exposed to increasing concentrations of gefitinib or dasatinib for 24 hours and total proteins were evaluated for phosphorylated Akt and STAT3 as well as cleaved PARP indicative of apoptosis (Fig. 2B; Supplementary Fig. S2B). In HCC827 cells, dasatinib inhibits pAkt and induces PARP cleavage at 50 nmol/L, whereas modest changes are observed in pSTAT3. These results are similar in gefitinib-treated HCC827 cells. In PC9 cells, dasatinib exerts a dose-dependent inhibition of pAkt with associated changes in PARP cleavage, whereas a 50 nmol/L dose of gefitinib completely inhibits pAkt and induces PARP cleavage. In H3255 cells, dasatinib results in a concentration-dependent decrease of both pAkt and pSTAT3 with corresponding increase in PARP cleavage, whereas

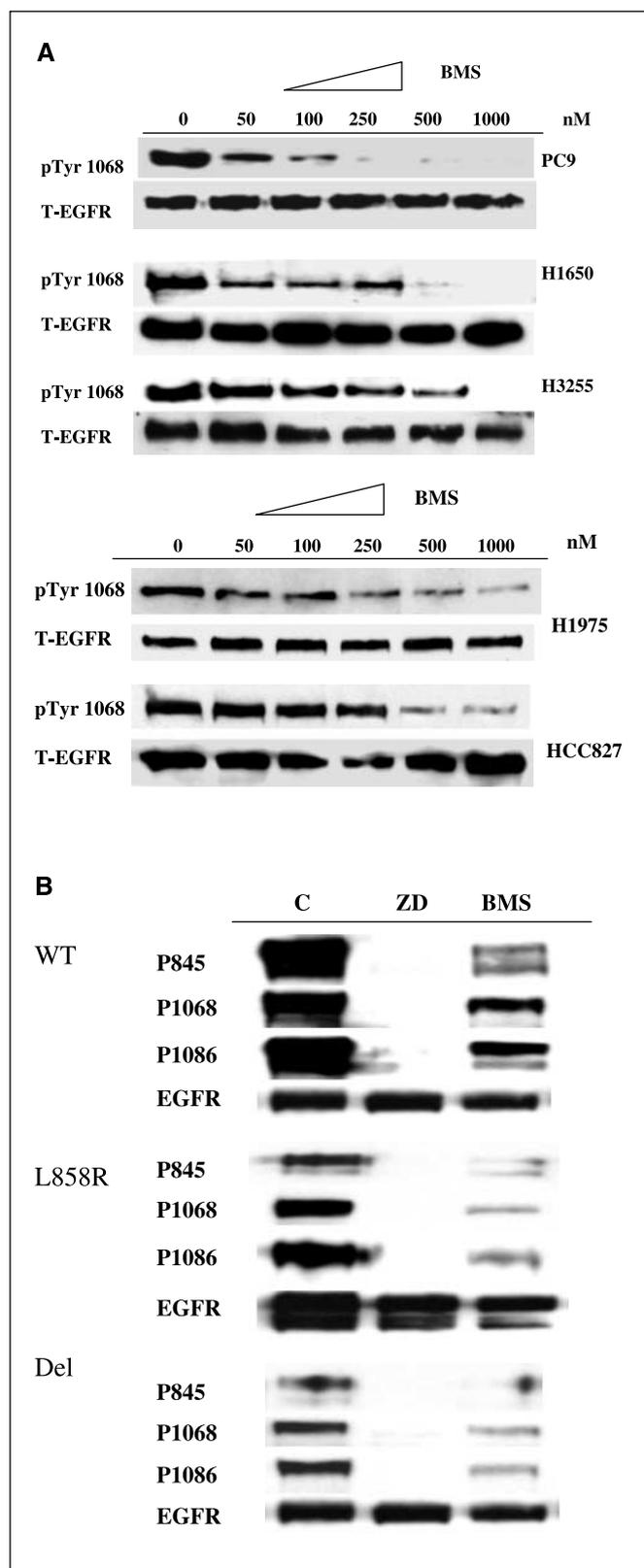


Figure 3. Effect of dasatinib and gefitinib on EGFR phosphorylation. **A**, cells were exposed to indicated concentrations of dasatinib, and total proteins were collected after 24 hours. Membranes were blotted with indicated antibodies. **B**, HEK293 cells were transfected with plasmids encoding indicated EGFR cDNA and exposed to either dasatinib (500 nmol/L) or gefitinib (1 μ mol/L) for 3 hours. Whole-cell lysates were prepared and subjected to Western analysis with indicated antibodies. **C**, control (DMSO).

a 50 nmol/L gefitinib completely inhibits both pAkt and pSTAT3 with associated induction of PARP cleavage. In H1650 cells grown in 5% BCS, dasatinib inhibits pAkt at 50 nmol/L with corresponding induction of PARP cleavage. Gefitinib has minimal effect on pAkt, and the degree of PARP cleavage is less corresponding to the higher IC_{50} of gefitinib under these growth conditions. When the same cells are grown in low serum with exogenous EGF, both dasatinib and gefitinib inhibit pAkt at 50 nmol/L, but again the degree of PARP cleavage is higher in dasatinib-treated cells. No effect of either dasatinib or gefitinib is seen on pSTAT3 in any growth conditions. Finally, neither gefitinib nor dasatinib affects pAkt or pSTAT3 in H1975 cells, and no PARP cleavage is observed. These studies show that the induction of apoptosis by dasatinib is associated with reduction in activated Akt or STAT3 in a manner similar to that of gefitinib, although, in some cells, higher concentrations are necessary to see the effect on signaling and apoptosis.

We next evaluated the effect of gefitinib and dasatinib on Src phosphorylation status (Fig. 2C). In HCC827 cells, dasatinib inhibits the lowest mobility pSrc band at 50 nmol/L, whereas no changes are seen in these cells with gefitinib. In PC9 cells, dasatinib inhibits pSrc at 50 nmol/L, whereas a decrease in pSrc is observed with 250 nmol/L gefitinib, but the effect is incomplete even at a 1 μ mol/L concentration. Dasatinib completely inhibits pSrc at 50 nmol/L in H1650 cells, whereas the effect with gefitinib is incomplete. In H1975 cells, dasatinib completely inhibits the low levels of pSrc at 50 nmol/L, whereas minimal changes are observed with gefitinib. These results suggest that, in these EGFR-mutant cells, pSrc is largely maintained through EGFR-independent mechanisms that can be overcome by dasatinib.

Because Src signaling has been shown to modify EGFR function and dasatinib has been suggested to bind EGFR, we next evaluated the effect of dasatinib on EGFR protein phosphorylation status (7, 8, 11). As shown in Fig. 3A, dasatinib induces a concentration-dependent inhibition of EGFR phosphorylation status in the cell lines evaluated. To confirm these results in another assay, we transfected HEK293 cells that have low endogenous Erb expression with expression plasmids encoding WT EGFR, L858R EGFR, and del L747-E749;A750P EGFR, exposed the cells to either gefitinib or dasatinib, and then evaluated activated EGFR using antibodies that specifically recognize distinct phosphotyrosines on EGFR (Fig. 3B; ref. 17). As expected, a 1 μ mol/L concentration of gefitinib completely inhibited EGFR phosphorylation, whereas a 500 nmol/L dose of dasatinib inhibited EGFR phosphorylation although not to the extent seen with gefitinib. These findings suggest that dasatinib can affect EGFR function possibly through a combination of direct binding and inhibition and/or indirectly through Src inhibition (7, 8, 10).

We next evaluated the effect of Src inhibition on lung cancer cells that do not have EGFR mutations and do not require EGFR for survival (Fig. 4). Dasatinib completely inhibits pSrc; however, a 1 μ mol/L dose of gefitinib was unable to inhibit pSrc in A549 or H358 cells with WT EGFR. In A549 cells, we observed a reduction in pFAK but no significant reductions in pSTAT3, pAkt, or pERK. Similar results were observed in H358 cells, although these cells have no observable pFAK. As shown in Fig. 4B, dasatinib results in cell cycle arrest characterized by increased G_1 fraction and reduced S-phase fraction in A549 cells despite no effect with gefitinib. We examined the effect of dasatinib on key regulatory molecules cyclins D1 and D3 and p27 involved in G_1 -S cell cycle progression that can be regulated by Src (6, 16). The G_1 block resulting from

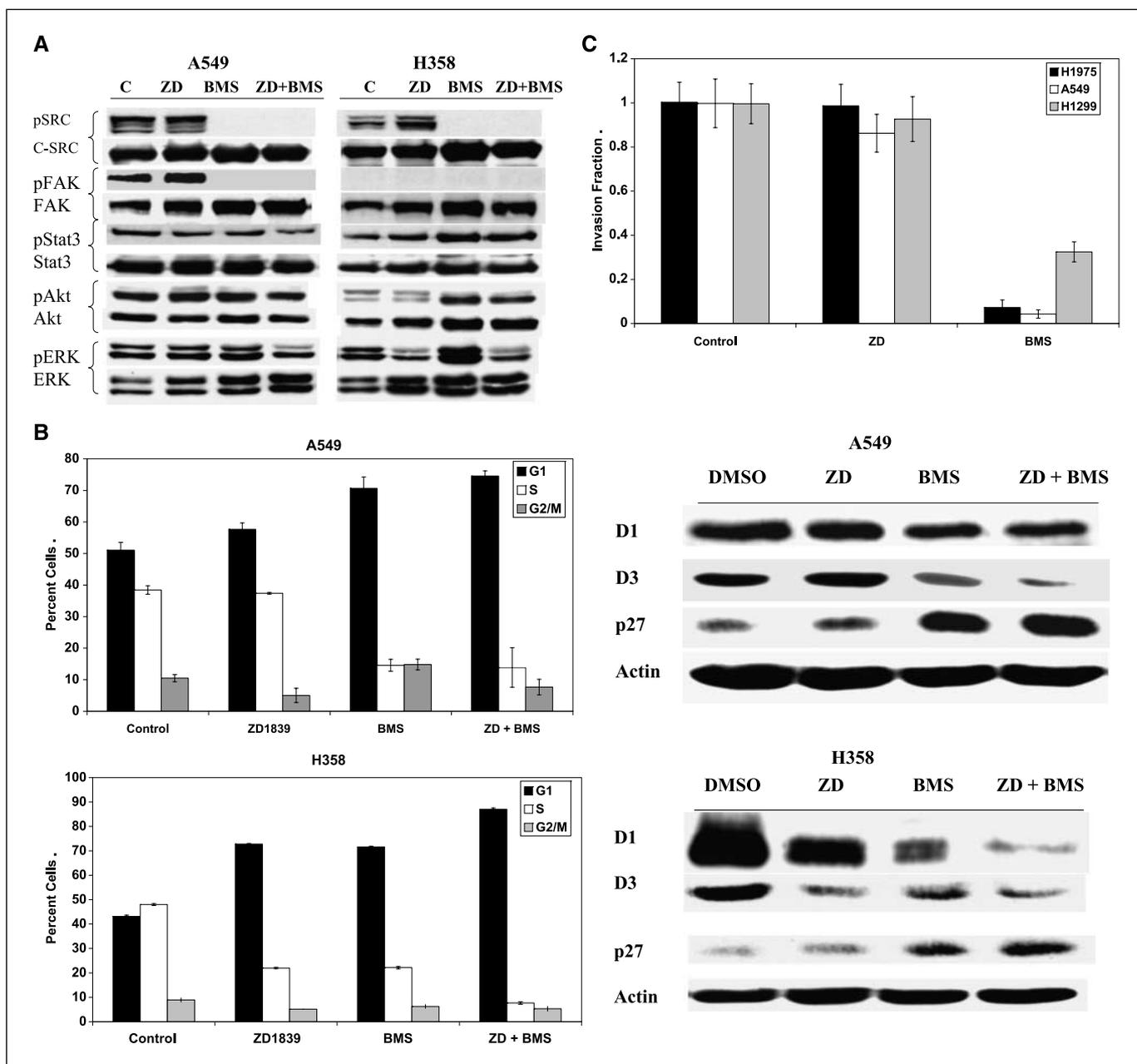


Figure 4. Comparative effect of dasatinib and gefitinib on cell cycle progression and tumor cell invasion. **A**, A549 and H358 cells were exposed to 1 $\mu\text{mol/L}$ gefitinib, 500 nmol/L dasatinib, or the combination (ZD+BMS) for 3 hours, and whole-cell lysates were subjected to Western analysis using indicated antibodies. **B**, A549 and H358 cells were exposed to 1 $\mu\text{mol/L}$ gefitinib, 500 nmol/L dasatinib, or the combination (ZD+BMS) for 24 hours, and cell cycle profiles were evaluated. Parallel group of cells was evaluated for changes in cyclins D1 and D3 and p27 by Western analysis. **C**, indicated cells were exposed to 1 $\mu\text{mol/L}$ gefitinib or 500 nmol/L dasatinib, and tumor cell invasion was quantified using Boyden chambers.

dasatinib is associated with a decline in cyclin D3 and an increase in p27, whereas no changes in these critical cell cycle proteins are observed in gefitinib-treated cells nor are further changes in cyclin D3 or p27 observed in cells treated with both agents. On the other hand, H358 cells undergo G₁ cell cycle arrest to the same extent between gefitinib and dasatinib, both compounds result in reduced cyclins D1 and D3 and increased p27 protein levels, and the combination results in further G₁ cell cycle arrest and more pronounced changes in cyclins D1 and D3 and p27. Finally, consistent with the known role for Src in regulating tumor cell invasion, dasatinib has a significant inhibitory effect on tumor cell

invasion in cells with WT EGFR (A549 and H1299) and gefitinib-resistant EGFR (H1975) mutation, whereas gefitinib has no effect compared with control (Fig. 4C; ref. 6).

Discussion

Our data suggest that the decision fork for apoptosis versus growth arrest in cells exposed to dasatinib is dependent on the degree of upstream EGFR dependence for survival. Dasatinib shuts down the EGFR-dependent survival network in a concentration-dependent manner and induces death in EGFR-dependent cells.

Our results agree with recent studies, showing dasatinib-induced apoptosis in head and neck cancer cells, another EGFR-dependent tumor type (18). More work is required to understand how dasatinib results in apoptosis in gefitinib-sensitive mutant EGFR lung cancer cells. In addition to Src proteins, dasatinib has been shown to bind other tyrosine kinase proteins, including EGFR, and, in conjunction with our data, suggests that EGFR may be a direct target of dasatinib or an indirect target secondary to Src inhibition (7, 8, 11). In addition, Src signaling can regulate the PI3K/PTEN/Akt axis through multiple mechanisms, including tyrosine phosphorylation of the regulatory p85 subunit of PI3K, tyrosine phosphorylation of PTEN that results in compromised function of PTEN, and modification of EGFR function through direct phosphorylation of key tyrosine residues (7, 16). Despite ample evidence that Src proteins can directly phosphorylate STAT3, only in H3255 cells we observed clear inhibition of pSTAT3 with dasatinib, and it is not clear if this is a direct effect on Src inhibition or rather through modification of EGFR function (9). Nonetheless, our results suggest that Src is not responsible for high levels of activated STAT3 seen in cells with EGFR mutation. It will be interesting to see if other Src tyrosine kinase inhibitors (TKI) produce similar effects on lung cancer cells with activating mutations in EGFR.

It will be important to examine the effect of dasatinib in additional cells with acquired resistance to EGFR-TKI. Despite dramatic responses in the subset of lung cancer patients with EGFR dependence, the tumor cells ultimately acquire resistance to EGFR-TKI therapy through either additional mutations in EGFR or other mechanisms (19). Multiple inhibitors may be necessary to overcome acquired resistance to TKIs in BCR-ABL-dependent leukemia, and a similar paradigm may exist in the treatment of EGFR-dependent lung cancer (20). Thus, combined attack on EGFR-dependent survival pathways by multiple nonoverlapping agents may be necessary to cure this subset of patients by avoiding the development of resistant clones. Although speculative,

dasatinib added to EGFR-TKI may help suppress development of resistant clones, but this obviously requires further testing. We found no apoptotic effect of dasatinib on H1975 cells with the T790M mutation, but this mechanism of resistance may be rare, and further evaluation in other cell lines that have acquired resistance to EGFR-TKI is indicated.

Dasatinib may have advantages over EGFR inhibitors in tumors that are not dependent on EGFR for survival through promoting tumor cell dormancy through cell cycle arrest and inhibition of tumor cell invasion. This is important because the majority of patients with advanced lung cancer do not have EGFR mutation. Because Src signaling is implicated in oncogenic processes, such as cell invasion, metastasis, and angiogenesis, these compounds could have additional *in vivo* effects beyond the effects seen in these cell culture models (6). Dasatinib is in early stages of clinical development in patients with solid tumors, and it remains unclear if patients can achieve dasatinib levels used in our studies that induce apoptosis, cell cycle arrest, and inhibition of invasion. A priori determination of lung cancers dependent on EGFR for growth and/or survival may identify patient subsets that derive the maximum benefit from dasatinib, and combination therapy with EGFR inhibitors should be considered. Further work in both animal models and clinical trials is indicated to validate this hypothesis.

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