Introduction

Insulin-like growth factor (IGF)-binding protein-2 (IGFBP2) expression is increased in various types of cancers, including in a subset of patients with lung cancer. Because IGFBP2 is involved in signal transduction of some critical cancer-related pathways, we analyzed the association between IGFBP2 and response to pathway-targeted agents in seven human non–small cell lung cancer (NSCLC) cell lines. Western blot analysis and ELISA showed that four of the seven NSCLC cell lines analyzed expressed high levels of IGFBP2, whereas the remaining three had barely detectable IGFBP2. Susceptibilities of those seven cell lines to nine anticancer agents targeting to IGF1R, Src, FAK, MEK, and AKT were determined by a dose-dependent cell viability assay. The results showed that high IGFBP2 levels were associated with resistance to dasatinib and, to a lesser degree, to sacaratinib, but not to other agents. Ectopic IGFBP2 overexpression or knockdown revealed that changing IGFBP2 expression levels reversed dasatinib susceptibility phenotype, suggesting a causal relationship between IGFBP2 expression and dasatinib resistance. Molecular characterization revealed that focal adhesion kinase (FAK) activation was associated with increased IGFBP2 expression and partially contributed to IGFBP2-mediated dasatinib resistance. Treatment with a combination of dasatinib and FAK inhibitor led to enhanced antitumor activity in IGFBP2-overexpressing and dasatinib-resistant NSCLC cells in vitro and in vivo. Our results showed that the IGFBP2/FAK pathway is causally associated with dasatinib resistance and may be used as biomarkers for identification of dasatinib responders among patients with lung cancer. Simultaneous targeting on Src and FAK will likely improve the therapeutic efficacy of dasatinib for treatment of lung cancer.

Abstract

Insulin-like growth factor (IGF)-binding protein-2 (IGFBP2) expression is increased in various types of cancers, including in a subset of patients with lung cancer. Because IGFBP2 is involved in signal transduction of some critical cancer-related pathways, we analyzed the association between IGFBP2 and response to pathway-targeted agents in seven human non–small cell lung cancer (NSCLC) cell lines. Western blot analysis and ELISA showed that four of the seven NSCLC cell lines analyzed expressed high levels of IGFBP2, whereas the remaining three had barely detectable IGFBP2. Susceptibilities of those seven cell lines to nine anticancer agents targeting to IGF1R, Src, FAK, MEK, and AKT were determined by a dose-dependent cell viability assay. The results showed that high IGFBP2 levels were associated with resistance to dasatinib and, to a lesser degree, to sacaratinib, but not to other agents. Ectopic IGFBP2 overexpression or knockdown revealed that changing IGFBP2 expression levels reversed dasatinib susceptibility phenotype, suggesting a causal relationship between IGFBP2 expression and dasatinib resistance. Molecular characterization revealed that focal adhesion kinase (FAK) activation was associated with increased IGFBP2 expression and partially contributed to IGFBP2-mediated dasatinib resistance. Treatment with a combination of dasatinib and FAK inhibitor led to enhanced antitumor activity in IGFBP2-overexpressing and dasatinib-resistant NSCLC cells in vitro and in vivo. Our results showed that the IGFBP2/FAK pathway is causally associated with dasatinib resistance and may be used as biomarkers for identification of dasatinib responders among patients with lung cancer. Simultaneous targeting on Src and FAK will likely improve the therapeutic efficacy of dasatinib for treatment of lung cancer.

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(ILK)/NF-κB network is reported to be a key player in progression and poor outcome of glioma (26). Increased expression of IGFBP2 promotes breast cancer cell metastasis by recruitment of endothelial cells to the cancer site through upregulation of the IGF/IGF1R signaling pathway (8).

Because both the IGF1R and integrin pathways frequently cooperate with other growth factor pathways in signal transduction of cancer cells (27, 28), we hypothesized that overexpression of IGFBP2 induces autocrine and/or paracrine effects through IGF1R, integrins, or both, thereby triggering alteration of cellular signal transduction and resistance to pathway-targeted therapy in lung cancer. To test whether overexpression of IGFBP2 induces resistance to targeted therapy in lung cancer cells, we evaluated responses to pathway-targeted anticancer agents in a panel of lung cancer cell lines with high or low IGFBP2 expression. Our results show that a high level of IGFBP2 expression in lung cancer cell lines is causally associated with increased phosphorylation of focal adhesion kinase (FAK) and resistance to dasatinib.

Materials and Methods

Chemicals and antibodies
Small-molecule IGFR1 inhibitors picropodophyllin, GSK 1904529A, BMS-754807, and OSI-906 (linsitinib) were obtained from Chemie Tek. Src inhibitors saracatinib and dasatinib were obtained from Selleck Chemical. PF-562271, a FAK inhibitor, was obtained from MedKoo Biosciences Inc. AZD6244, a mitogen-activated protein kinase kinase (MEK) inhibitor, and MK2206, an Akt inhibitor, were obtained from the Translational and Analytical Chemistry Core facility of The University of Texas MD Anderson Cancer Center (Houston, TX). Antibodies for total and phosphorylated FAK (p-FAK; pY397) were purchased from Epitomics. Antibodies for IGFBP2, total Src, phosphorylated Src (p-Src; Y527 and Y416), total Akt, and phosphorylated Akt (p-Akt; S473) were obtained from Cell Signaling Technology. ILK antibody and IGFBP2 DuoSet ELISA kit were obtained from R&D Systems. Protease inhibitor cocktail, β-actin antibody, and sulforhodamine were obtained from Sigma Chemical Corporation. Protein assay reagents were purchased from Bio-Rad Laboratories.

Cell lines and cell culture
Human non–small cell lung cancer (NSCLC) cell lines were maintained in our laboratories as previously described (29). The authentication for each cell line was conducted by short tandem repeat–based DNA fingerprint analysis within 12 month. The cells were cultured in RPMI-1640 or high-glucose Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS, 100 μg/mL ampicillin, and 0.1 mg/mL streptomycin; they were maintained at 37°C in a humidified atmosphere containing 5% CO2 and 95% air.

Cell viability assays
The inhibitory effects of pathway-targeted anticancer agents on cell growth were determined by using the sulforhodamine B assay, as described previously (29). Each experiment was carried out in quadruplicate and repeated at least 3 times. The relative cell viability (%) was calculated using the equation ODf/ODc × 100% (where ODf represents the absorbance of the treatment group and ODc represents the absorbance of the control group). The median inhibitory concentration (IC50) values were calculated by using CurveExpert 1.3 software.

Western blot analysis
Whole-cell lysates were prepared by washing the cells with PBS and subjecting them to lysis with radioimmunoprecipitation assay (RIPA) buffer supplemented with the protease inhibitor cocktail. After the lysates were sonicated for 15 seconds, the protein concentrations were quantified using the Bio-Rad Protein Assay Kit. Equivalent amounts of each protein were loaded, separated by 10% or 12% SDS-PAGE and then transferred to polyvinylidene fluoride membranes at 80 V for 2 hours. The membranes were blocked for 1 hour with 5% nonfat dried milk in PBS buffer containing 0.1% Tween-20 (PBST) and probed with diluted primary antibody at 4°C overnight. The membranes were then washed 3 times in the PBST buffer and probed with infrared dye–labeled secondary antibodies. The immunoreactive bands were visualized with the Odyssey Imager (Li-COR Biosciences).

ELISA for IGFBP2
IGFBP2 concentrations in media from cell cultures were determined with the IGFBP2 DuoSet ELISA kit according to the manufacturer’s protocol. For this purpose, cells (5 × 104 per well) were cultured in 24-well plates with 0.5 mL of medium for 48 hours. The ELISA capture antibody was diluted to 6 μg/mL in 0.1 mol/L NaHCO3, pH 9.5, and coated on 96-well ELISA plates. After incubation overnight at room temperature, the plates were washed in TBS buffer (50 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 0.05% Tween-20) and blocked with 1% bovine serum albumin. Culture medium supernatant (100 μL) or IGFBP2 standard was added to each plate and the plates were incubated for 2 hours at room temperature, followed by immunoreaction with horseradish peroxidase–labeled IGFBP2 detection antibody and colorimetric detection with the substrate 3,3′,5′,5′-tetramethyl benzidine dihydrochloride (Sigma). IGFBP2 concentration was calculated from the standard curve. All experiments were carried out in duplicate and repeated twice.

Lentiviral vector-mediated gene knockdown or overexpression
Plasmids for lentiviral vectors expressing cDNA or short hairpin RNA (shRNA) used in this study were obtained from Open Biosystems through the shRNA and ORFeome Core facility in The University of Texas MD Anderson Cancer Center. Lentiviral vectors were
packaged in 293 cells after cotransfection with lentiviral packaging plasmids by using Fugene 6 as instructed by the manufacturer (Promega). The medium from transfected 293 cells was filtered through a sterile 0.22-μm filter and used to infect target cells in the presence of 8 μg/mL polybrene. After selection with puromycin (1–2 μg/mL) or blasticidin (2–10 μg/mL), the cells were pooled together for subsequent studies. The proportion of stably transfected cells (expressing GFP or RFP in the lentiviral backbone) was usually more than 85% after selection.

Animal experiments
Animal experiments were carried out in accordance with Guidelines for the Care and Use of Laboratory Animals (NIH publication number 85-23) and the institutional guidelines of MD Anderson Cancer Center. Subcutaneous tumors were established in 6- to 8-week-old female nude mice (Charles River Laboratories Inc.) by inoculation of 2 × 10^7 H460 cells into the dorsal flank of each mouse. After the tumors grew to 3 to 5 mm in diameter, the mice were grouped randomly into four groups and treated with oral administration of (i) dasatinib (25 mg/kg/d), (ii) PF-562271 (25 mg/kg/d), (iii) both dasatinib and PF-562271 as in the groups 1 and 2, and (iv) solvent (10% dimethyl sulfoxide and 10% polyethylene glycol 400). Tumor volumes were calculated by using the formula \( V = \frac{a \times b^2}{2} \), where \( a \) and \( b \) represented the larger and smaller diameters, respectively. Mice were killed when the tumors grew to 15 mm in diameter. Blood samples were collected from the tail vein one day after the last treatment, and serum alanine transaminase, aspartate transaminase, and IgG levels were determined at the Research Animal Support Facility of our institution.

Statistical analysis
Each experiment or assay was conducted at least 2 times, and representative examples are shown. Data are reported as mean ± SD or SE. Statistical significance of the differences between treated samples was determined by using the two-tailed Student \( t \) test and one-way ANOVA analysis. Differences were considered statistically significant at \( P < 0.05 \).

Results
IGFBP2 expression in lung cancer cells is associated with sensitivity to dasatinib and saracatinib
Our recent study showed that expression of IGFBP2 is dramatically increased in some primary lung cancer tissues (17). To test whether IGFBP2 is also increased in cultured lung cancer cell lines, we conducted Western blot analysis on seven NSCLC cell lines. IGFBP2 was highly expressed in Calu3, H460, H1437, and H3122 cells but was barely detectable in H1299, H1792, and H1944 cells (Fig. 1A). Moreover, ELISA detected high levels of IGFBP2 (≥ 150 ng/mL) in the culture media collected from Calu3, H460, H1437, and H3122 cells, whereas IGFBP2 was barely detectable in the media collected from H1299, H1792, and H1944 cells (Fig. 1B). Together, those results show that IGFBP2 is differentially expressed in NSCLC cell lines.

Because IGFBP2 can modulate functions of the IGFIR and integrin pathways, both of which have been explored as targets for anticaner therapy and/or implicated in the mechanisms of other pathway-targeted therapies (27, 30), we analyzed responses to various clinically relevant pathway-targeted anticancer agents in the same seven NSCLC lung cancer cell lines, which express either high or low levels of IGFBP2. The anticancer agents used are listed in Materials and Methods. The antitumor activity of each agent at various doses ranging from 0.03 to 30 μmol/L was determined by a cell viability assay. The \( IC_{50} \) of each agent in each cell type was calculated from the dose–response curve. The results show that the level of IGFBP2 expression in NSCLC cell lines was not associated with responses to most of the agents tested; the exceptions were dasatinib and saracatinib (Table 1). The NSCLC cell lines expressing high levels of IGFBP2 were highly resistant to dasatinib, whereas the cell lines expressing low levels of IGFBP2 were quite sensitive to dasatinib (Fig. 1C). This relationship was also observed for saracatinib, although this agent was less active in the sensitive cells. In contrast, there was no noticeable correlation between IGFBP2 expression level and responses to other anticancer agents (Table 1). The dose responses for BMS-754807 and PF562271 are shown in Fig. 1C and D, respectively, as examples. The inverse association of IGFBP2 expression with sensitivity to dasatinib observed in this study is consistent with a published report showing that global gene expression profiling analysis identified IGFBP2 as one of six genes that can effectively predict \textit{in vitro} dasatinib response in breast cancer and lung cancer cell lines (31).

IGFBP2 overexpression is causally related to resistance to dasatinib
A recent study showed that kinase-impaired \textit{B}Raf mutation is associated with dasatinib sensitivity in some NSCLC cells (32). Nevertheless, the gene mutation status of the cell lines used in the present study, obtained from the Sanger Institute Catalogue Of Somatic Mutations In Cancer web site, \texttt{http://cancer.sanger.ac.uk/cancergenome/projects/cosmic}, and our lung cancer database, showed that all cell lines tested except H3122, for which \textit{B}Raf mutation status is not known, have the wild-type \textit{B}Raf gene (Table 1). There is no obvious association between dasatinib sensitivity and mutation in the \textit{K}Ras, \textit{N}Ras, \textit{E}GF\textit{R}, or \textit{p}53 genes.

We then investigated whether overexpression of IGFBP2 was the culprit in resistance to dasatinib. To this end, we stably transfected H1299 cells with a lentiviral vector expressing IGFBP2 or a control lentiviral vector. The expression of IGFBP2 in H1299/IGFBP2 cells, but not in parental H1299 or H1299/vector cells, was confirmed by Western blot analysis of cell lysates and ELISA of the culture media collected from those cells (Fig. 2A and B). Cell viability analysis showed that ectopic expression of
IGFBP2 in H1299 resulted in resistance to dasatinib (Fig. 2C). The IC50 was more than 100-fold greater in H1299/IGFBP2 cells than in parental or vector-transfected H1299 cells, suggesting that IGFBP2 alone is sufficient to induce resistance to dasatinib.

To further validate a role for IGFBP2 in the response to dasatinib, we knocked down IGFBP2 in H1437 and Calu3 cells through lentiviral vector–mediated expression of IGFBP2 shRNA. The knockdown of IGFBP2 was confirmed by Western blot and ELISA analyses (Fig. 2D, E, G, and H). The cell viability assay showed that knockdown of IGFBP2 in H1437 and Calu3 cells was sufficient to sensitize these cells to dasatinib (Fig. 2F and I). Whereas the dasatinib IC50 was ≥4.5 μmol/L for parental and control shRNA-transfected H1437 and Calu3 cells, it was about 0.07 and 0.006 μmol/L, respectively, in the IGFBP2-knockdown cells. In contrast, lentiviral vector–mediated knockdown of Src had no effect on dasatinib dose response in Calu3 cells (data not shown). Together, these results show that cellular IGFBP2 levels were causally associated with dasatinib sensitivity in NSCLC cells.

**IGFBP2 overexpression induces FAK phosphorylation**

To identify pathways or downstream mediators that might have been associated with IGFBP2 expression and dasatinib susceptibility in the NSCLC, we analyzed the expression and/or phosphorylation of several key IGFBP2 downstream molecules reported in literature. Among them were IGF1R (8), Akt (34), ILK (26), FAK, and Src. Lysates from the seven NSCLC cell lines were analyzed for expression and/or phosphorylation of these molecules.
proteins. The results show that, of the molecules tested, only the pattern of FAK phosphorylation (p-Y397) was consistent with IGFBP2 expression in the seven NSCLC cell lines (Fig. 3A). All four cell lines expressing high levels of IGFBP2 had high levels of p-FAK, whereas all 3 of the cell lines expressing low levels of IGFBP2 had low levels of p-FAK, strongly suggesting a functional association between IGFBP2 expression and FAK phosphorylation.

We then tested whether ectopic expression of IGFBP2 in H1299 cells and knockdown of IGFBP2 in Calu3 and H1437 cells would elicit changes of FAK phosphorylation in those cells. Western blot analysis revealed that ectopic expression of IGFBP2 in H1299 substantially increased the levels of FAK phosphorylation, whereas knockdown of IGFBP2 in Calu3 and H1437 cells reduced FAK phosphorylation (Fig. 3B and C) and also suggest that FAK may be a potential downstream mediator of IGFBP2.

**FAK activity is associated with dasatinib resistance**

To characterize the possible roles of cellular FAK activity in dasatinib resistance, we stimulated FAK activity by using fibronectin as reported elsewhere (35). H1299 and H1972 cells were cultured in plates coated with fibronectin and then harvested for Western blot analysis. The results show that culturing those cells in the fibronectin-coated plates led to dramatic increases in phosphorylated FAK in those cells (Fig. 4A). The cell viability assay showed that cells cultured in the fibronectin-coated plates were less sensitive to dasatinib than the cells cultured in uncoated plates (Fig. 4B and C). This result suggests, at least, that increased FAK activity partially contributes to resistance to dasatinib in NSCLC cells.

To further examine the role of FAK in dasatinib susceptibility of NSCLC, we determined the combined effects of dasatinib and a FAK inhibitor in the dasatinib-resistant NSCLC. We first tested the dose response to dasatinib in Calu3, H460, H1437, and H3122 cells in the presence or absence of PF562271 (1 μmol/L). While PF562271 at this concentration had only mild effects (14%–28% reduction) on cell viability in those cells, its presence dramatically shifted the dose-response curves and enhanced the response to dasatinib in all four cell lines. On the basis of the dose response to single-agent dasatinib or PF562271, as shown in Fig. 1 and Table 1, we conducted cell viability analysis for the combined effects of dasatinib and PF562271 at various concentrations. The ratio of the 2 compounds was based on the IC_{50} value of each. The cell viability assay was conducted in parallel in cells treated with each single agent. The combined effect of the 2 agents was determined by calculating the combination index according to the Chou–Talalay method (36), using CalcuSyn software (Biosoft) as we reported previously (37). The results show that the combination led to synergistic or additive effects at various dasatinib:PF562271 ratios ranging from 1:1 to 3:1 (Fig. 5A–C, Supplementary Table S1). This result suggests that for the cell lines having high levels of IGFBP2 and FAK phosphorylation that were resistant to dasatinib, treatment with a combination of dasatinib and FAK inhibitor would improve the therapeutic effects.

We also tested the combination effects of dasatinib and PF562271 in vivo in a xenograft tumor model–derived H460 cells. Dasatinib (25 mg/kg) and PF562271 (25 mg/kg) alone or in combination were administrated daily to mice bearing H460 tumors. Animals treated with solvent were used as controls. The tumor volumes were monitored during the treatment. The results showed that there was no obvious difference in tumor growth between control and PF562271-treated animals. Treatment with dasatinib resulted in a mild but insignificant suppression of H460 tumors. Nevertheless, the combination led to significant growth suppression of H460 tumors in vivo (Fig. 5D). The results show that the combination therapy is effective and well tolerated.

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**Table 1. IC_{50} of nine anticancer agents and gene mutation data in seven NSCLC cell lines**

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<th>Compounds</th>
<th>Targets</th>
<th>H1792 IC_{50} (μmol/L)</th>
<th>H1944 IC_{50} (μmol/L)</th>
<th>H1299 IC_{50} (μmol/L)</th>
<th>Calu3 IC_{50} (μmol/L)</th>
<th>H460 IC_{50} (μmol/L)</th>
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**Mutation status**

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Discussion

The drastic increase of IGFBP2 expression in a subset of lung cancer tissues and potential roles of IGFBP2 in signal transduction in cancer cells led us to investigate whether overexpression of IGFBP2 is associated with response to pathway-targeted therapy in lung cancer. Our results show that IGFBP2 overexpression is causally associated with resistance to dasatinib in NSCLC and that FAK, a downstream signaling molecule of IGFBP2, may contribute to dasatinib resistance.

Dasatinib was initially identified as a dual Src/Abl kinase inhibitor with potent antitumor activity (38), but subsequently it was found to inhibit multiple kinases, including Src family kinase members (LYN, SRC, FYN, LCK, and YES), nonreceptor tyrosine kinases (FRK, BRK, and ACK), and receptor tyrosine kinases (ephrin receptors, DDR1, EGFR, and PDGFR; ref. 39). Dasatinib is currently approved for treatment of chronic myeloid leukemia (40) and has been investigated in clinical trials for treatment of lung cancer (41–43). The results from phase I and II trials suggest that a subpopulation of patients with lung cancer will benefit from treatment with dasatinib and that a biomarker-directed personalized strategy will be required for the success of future studies (41–43). Thus, identification of biomarkers that are associated with dasatinib susceptibility in lung cancer will...
have an impact on the future success of treating patients with lung cancer with this agent.

Effort has been made to identify molecular markers associated with dasatinib susceptibility in lung cancer cells. Recent studies showed that mutations in BRaf (32) and DDR2 (44) may render lung cancer cells susceptible to dasatinib. As all cell lines used in this study are BRaf wild-type, the susceptibility differences observed in those cell lines are unlikely to be associated with BRaf mutations. Because we do not have data on DDR2 gene mutations in our lung cancer cell lines, it is unclear whether DDR2 mutations contribute to the dasatinib susceptibility in those cell lines. Nevertheless, the ectopic gene overexpression and gene knockdown experiments in the present study show that IGFBP2 level is causally associated with resistance to dasatinib in NSCLC. This observation is consistent with a previous report by others that IGFBP2 is one of six genes whose levels in breast and lung cancer cell lines could predict cancer cells’ response to dasatinib (31). Interestingly, IGFBP2 was also found to be associated with susceptibility to saracatinib, another Src inhibitor, in pancreatic cancer (45). We observed a similar tendency for an association between IGFBP2 level and saracatinib susceptibility in our study, although saracatinib was less active than dasatinib in dasatinib-sensitive NSCLC.

We also found that FAK is a candidate downstream mediator in IGFBP2-mediated signaling. A, basal levels of Src, p-Src, FAK, p-FAK, AKT, p-AKT, and ILK were determined in the seven NSCLC cell lines shown in Fig. 2. Level of p-FAK was high in all four cell lines that expressed high levels of IGFBP2. B and C, changes in p-FAK levels were measured in H1299, Calu3, and H1437 cells after ectopic expression or knockdown of IGFBP2 as described for Fig. 2. Notably, p-FAK increased dramatically in IGFBP2-overexpressing H1299 cells and decreased dramatically in IGFBP2-knockdown Calu3 and H1437 cells.
upstream kinases Src and Lyn (49, 50). Nevertheless, our results indicate that elevated IGFBP2 expression and activated FAK in cancer cells are causally associated with dasatinib resistance and that combination of dasatinib with a FAK inhibitor may overcome this resistance. These results may have an impact on design of future clinical trials to identify effective regimens combining these innovative anticancer agents for the treatment of lung cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H. Lu, W.L. Hofstetter, B. Fang
Development of methodology: H. Lu, W.L. Hofstetter
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Lu, L. Wang, W. Gao, B. Dai, W.L. Hofstetter, S.G. Swisher
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Lu, W. Gao, J. Meng, S. Wu, J. Minna, J.A. Roth, B. Fang
Writing, review, and/or revision of the manuscript: H. Lu, J. Minna, J.A. Roth, W.L. Hofstetter, S.G. Swisher, B. Fang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Lu, L. Wang, J.A. Roth, W.L. Hofstetter
Study supervision: H. Lu, W.L. Hofstetter, B. Fang
Provided grant support for the project: J. Minna

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Figure 5. Effect of FAK inhibitor on dasatinib-mediated antitumor activity. A, H460 and H1437 cells were treated with FAK inhibitor PF562271 (1 μmol/L) for the times indicated. p-FAK and FAK levels were determined by Western blot analysis. B, dose responses of Calu3, H460, H1437, and H3122 cells to dasatinib in the absence or presence (+) of 1 μmol/L PF562271 were plotted. PF562271 alone had only mild effects on cell viability (cell viability about 72%–86% in those cells). C, dose responses of H1437 cells to dasatinib as a single agent and combined with PF562271. Synergistic effects were detected for the combination therapy. D, in vivo growth of H460 tumors. The mice were treated as indicated. The values are means ± SE of data from 5 mice per group. *, P < 0.05 when compared with other groups.
References


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