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Kinase Impaired *BRAF* Mutations Confer Lung Cancer Sensitivity to Dasatinib

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Abstract

During a clinical trial of the tyrosine kinase inhibitor dasatinib for advanced non–small cell lung cancer (NSCLC) one patient responded dramatically and remains cancer-free 4 years later. A comprehensive analysis of his tumor revealed a previously undescribed, kinase inactivating *BRAF* mutation (^{Y472C}*BRAF*); no inactivating *BRAF* mutations were found in the non-responding tumors taken from other patients. Cells transfected with ^{Y472C}*BRAF* exhibited CRAF, MEK, and ERK activation – characteristics identical to signaling changes that occur with previously known kinase inactivating *BRAF* mutants. Dasatinib selectively induced senescence in NSCLC cells with inactivating *BRAF* mutations. Transfection of other NSCLC cells with these *BRAF* mutations also increased these cells' dasatinib sensitivity, whereas transfection with an activating *BRAF* mutation led to their increased dasatinib resistance. The sensitivity induced by ^{Y472C}*BRAF* was reversed by the introduction of a *BRAF* mutation that impairs RAF dimerization. Dasatinib inhibited CRAF modestly, but concurrently induced RAF dimerization resulting in ERK activation in NSCLC cells with kinase inactivating *BRAF* mutations. The sensitivity of NSCLC with kinase impaired *BRAF* to dasatinib suggested synthetic lethality of BRAF and a dasatinib target. Inhibiting BRAF in NSCLC cells expressing wild-type *BRAF* likewise enhanced these cells' dasatinib sensitivity. Thus, the patient's *BRAF* mutation was likely responsible for his tumor's marked response to dasatinib, suggesting that tumors bearing kinase impaired *BRAF* mutations may be exquisitely sensitive to dasatinib. Moreover, the potential synthetic lethality of combination therapy including dasatinib and BRAF inhibitors may lead to additional therapeutic options against cancers with wild-type *BRAF*.

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INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide. Researchers recently identified two molecular subpopulations of non-small cell lung cancer (NSCLC)—populations with *epidermal growth factor receptor* (*EGFR*) mutations and those with EML4-ALK translocations—for which treatment with targeted agents has produced profound clinical responses. This development has elicited a paradigm shift in treating lung cancer: the tumors' genetic characteristics dictate therapy. Nevertheless, definition of clinically relevant genetic aberrations is lacking in about 85% of NSCLC cases.

One approach to defining these determinants and thus to discovering effective therapeutic targets is by characterizing tumors that respond significantly to targeted agents. This approach led to the discovery of an increased response to EGFR inhibitors in patients with NSCLC harboring *EGFR*-activating mutations in an initial characterization of nine patients (1). In a Phase 2 study of the multitargeted kinase inhibitor dasatinib in 34 patients with systemic therapy-naïve stage IV NSCLC, we observed in one patient at 12 weeks of treatment an initial partial response that continued to improve long after dasatinib was discontinued for toxicity (malaise), suggesting that dasatinib induced cancer cell senescence (2). This patient [patient X (PX)], received no further therapy and at present is alive and free of active cancer 4 years after his initial diagnosis.

We hypothesized that a comprehensive analysis of PX's tumor would identify the determinants of his remarkable response to dasatinib. We thus undertook a comprehensive analysis of his tumor that included mutational analysis of 40 genes, array comparative genomic hybridization, and immunohistochemistry and used site-directed mutagenesis, kinase assays, cell-cycle analysis, TUNEL, and β -galactosidase staining in vitro in NSCLC cell lines to characterize a mutation identified in the tumor. We discovered a previously undescribed *BRAF* mutation, ^{Y472C}*BRAF*, that results in substantial impairment of BRAF kinase activity. BRAF is a key factor in the RAS pathway: once activated by surface receptors, RAS activates RAFs, including BRAF. RAF phosphorylates mitogen-activated protein kinase kinase (MEK), which then activates extracellular signal-regulated kinase (ERK), which can promote cancer progression or induce cell senescence (3). *BRAF* mutations include those that cause kinase activation or impair kinase activity. Paradoxically, most *BRAF* mutants with reduced kinase activity still activate MEK and ERK via transactivation of CRAF (4, 5). In the study described herein, we tested whether the marked and durable clinical response of our patient was due to dasatinib-induced cancer cell senescence of ^{Y472C}*BRAF*-carrying cells.

RESULTS

Patients' Tumor Analysis

In our Phase 2 study of dasatinib in 34 patients with systemic therapy-naïve stage IV NSCLC the sole responder was a male former smoker (PX) who had a profound, durable response (2). Over the 12 weeks of dasatinib-based therapy, PX had a partial response as assessed by both tumor size and metabolic activity and his metastatic tumor (in paraspinal muscle) continued to shrink after therapy was stopped. At the end of therapy, the diameter of the metastasis was 2.8 cm, with a standardized uptake value (SUV) of 17. At 17 weeks, accurately measuring the metastasis on a computed tomography (CT) scan was difficult, but the SUV was 11. At 21 weeks, the SUV was 4.5. At 32 weeks, the mass was undetectable on CT and positron emission tomography scans (2). Subsequent follow up shows that PX remains free of active cancer 4 years after the initial diagnosis and has not received any other cancer therapy. PX still has a 2-cm lung nodule that has no detectable metabolic activity on PET and that has been stable on CT scans for 4 years (Figure S1A). The median

progression free survival was 1.4 months and the median overall survival was 15.6 months (Figure S1B). We performed additional studies of PX's tumor tissue to identify the underlying mechanism of dasatinib sensitivity.

PX's tumor did not harbor any *EGFR* or *KRAS* mutations by intron-based polymerase chain reaction (PCR) of *K-Ras* exons 1 and 2 (codons 12, 13, and 61) and *EGFR* exons 18–21 as previously published (2). We did not detect any *ALK* gene rearrangements by fluorescence in situ hybridization; *c-Src* mutations by intron-based PCR of exons 7–10; nor any *discoidin domain receptor 2 (DDR2)* mutations using conventional Sanger Sequencing of all exons as previously described (6). Immunohistochemistry revealed that the tumor did express total and phosphorylated EphA2, c-Kit, and PDGFR (Table S1). PX did not harbor any germ line *BRAF* or *KRAS* mutations by intron-based PCR of *BRAF* exons 11 and 15 and *K-Ras* exons 1 and 2 (codons 12, 13, and 61) of DNA isolated from his peripheral blood lymphocytes.

To identify novel mutations or changes in gene copy number in PX's tumor, we used the MassARRAY system (Sequenom) and performed aCGH. We identified no mutations among the 40 genes tested (Table S2). Using aCGH, we identified several regions of increased and decreased copy numbers (Figure S2; Table S3). We also observed increased copy numbers of the known direct dasatinib targets HCK, DDR1, EPHA3, and ARG (ABL2). We found no copy number changes for LYN, FGR, FYN, SRC, DDR2, EPHB1, EPHB2, EPHB3, EPHA1, EPHA2, EPHA4, TNK2, PTK6, GAK, KIT, PDGFR, KRAS, EGFR, or BRAF.

Identification of a Novel Inactivating Mutation in BRAF

Because the Sequenom MassARRAY technology is limited in that can only identify candidate mutations in which assays are specifically designed and given the known role of BRAF in oncogene-induced senescence, we sequenced exons 11 and 15 of *BRAF*. These two exons possess many known mutations not included in our panel. We identified the mutation ^{Y472C}*BRAF*, which has not been described previously and occurs in a highly conserved region of exon 11 (Figures S3 and S4). We also analyzed *BRAF* in 19 patients from our original clinical trial for whom DNA adequate for analysis was available and found no other inactivating mutations (Table S4).

To determine the functional significance of ^{Y472C}*BRAF*, we used site-directed mutagenesis to create ^{Y472C}*BRAF*, ^{G466V}*BRAF* (kinase-impaired), and ^{V600E}*BRAF* (constitutively active) in a Flag-tagged ^{wt}*BRAF* construct. We transfected the constructs into COS7 cells, isolated the Flag-tagged proteins and tested for kinase activity. As expected, ^{V600E}*BRAF* had increased kinase activity and ^{G466V}*BRAF* had reduced kinase activity. ^{Y472C}*BRAF* showed severely reduced kinase activity that was less than 10% that of ^{wt}*BRAF* (Figure 1A).

Lung Cancer Cells Expressing ^{Y472C}*BRAF* Express Activated MEK, ERK, and CRAF

Cancer cells that express kinase impaired BRAF mutations still activate MEK and ERK via transactivation of CRAF (4, 5). To determine whether ^{Y472C}*BRAF* functions similarly, we transfected COS7, H226, and H661 cells (all with ^{wt}*BRAF*) with the *BRAF* construct panel and measured their ERK and MEK activity (Figures 1B–1D). As we expected, transfection with ^{V600E}*BRAF* resulted in phosphorylation of ERK and MEK to levels markedly above those in cells transfected with ^{wt}*BRAF*. ^{Y472C}*BRAF* and ^{G466V}*BRAF* both activated MEK and ERK to levels at or above those observed after transfection with ^{wt}*BRAF*. Cells transfected with ^{wt}*BRAF* had similar levels of total BRAF compared to cells transfected with mutant *BRAF* (Figures 1C and 1D).

To determine whether ^{Y472C}*BRAF* transactivates CRAF, we assayed for CRAF kinase activity in H661 cells transfected with our *BRAF* constructs panel. Cells expressing ^{G466V}*BRAF* or ^{Y472C}*BRAF* had higher CRAF activity than did those cells expressing ^{wt}*BRAF* or ^{V600E}*BRAF* (Figure 1E). Likewise, cells expressing ^{G466V}*BRAF* or ^{Y472C}*BRAF* had higher BRAF-CRAF binding than did cells expressing ^{wt}*BRAF* or ^{V600E}*BRAF* (Figure 1F). These results confirmed that ^{Y472C}*BRAF* functions in the RAS/RAF/MEK/ERK pathway similarly to previously characterized BRAF proteins with inactivating mutations.

Dasatinib Leads to Senescence and Apoptosis in Lung Cancer Cells Expressing Kinase impaired BRAF

To determine whether ^{Y472C}*BRAF* was related to PX's response to dasatinib, we tested a panel of NSCLC cell lines for their sensitivity to dasatinib. After extensive searching in multiple databases, we identified only two NSCLC cell lines with known inactivating *BRAF* mutations: H1666 and Cal12T. These two lines, along with one cell line expressing ^{wt}*BRAF* (H322), were sensitive to dasatinib (Table 1). Researchers previously characterized H322 cells as having *c-Src* amplification, which drives their sensitivity to dasatinib (7). All other lines tested were resistant to dasatinib, including those with activating *BRAF* mutations (Table 1).

We did not observe significant apoptosis when we treated H1666 or Cal12T cells with dasatinib (Figure S5), but the cells did undergo cell-cycle arrest with an increased proportion of cells in G1, decreased proliferation with reduced BrdU incorporation, and they stained for -galactosidase and HPI-gamma, indicating senescence (Figures 2A–2D and S6). Consistent with this finding, dasatinib reduced phosphorylated Rb in H1666 and Cal12T (Figures 2E).

To determine whether dasatinib-induced senescence was reversible, we incubated Cal12T and H1666 cells with dasatinib for 6–96 h followed by drug removal and measurement of -galactosidase expression (Figure 2F, S7). Induction of significant tumor cell senescence required 72 h of exposure to dasatinib and was largely irreversible by 96h.

To further study the effects of inactivating *BRAF* mutations on dasatinib sensitivity, we transfected H661 and H226 cells with the panel of *BRAF* constructs. Expression of the inactivating mutations increased the cells' sensitivity to dasatinib, whereas expression of ^{V600E}*BRAF* induced further resistance (Figures 3A, 3B, and S8). In contrast H1666 and Cal12T cells, which have endogenous *BRAF* inactivation (^{G466V}*BRAF*), H661 cells, which we transfected with kinase impaired *BRAF*, underwent apoptosis and not senescence when exposed to dasatinib (Figure 3C).

To confirm the importance of the inactivating *BRAF* mutations in mediating dasatinib sensitivity in H1666 cells, we transfected these cells with kinase active *BRAF* (i.e., ^{V600E}*BRAF* or ^{wt}*BRAF*) and treated them with dasatinib (Figure 3D). Overexpression of kinase active BRAF increased resistance to dasatinib, confirming the role of kinase inactive BRAF in mediating dasatinib sensitivity. None of the constructs in the panel of *BRAF* constructs had a significant effect on cell number in untreated cells (Figure S9).

Dasatinib Indirectly Inhibits CRAF

The mechanism by which dasatinib induces senescence in NSCLC cells with kinase-deficient BRAF is unknown. We hypothesized that dasatinib induces senescence by affecting CRAF function (8). Although we found that dasatinib did not directly affect CRAF or BRAF kinase activity at relevant concentrations (Figure 4A), which is consistent with published studies (7, 9), it led to decreased CRAF activity in intact cells that express kinase inactive BRAF (Figure 4B). To establish the importance of CRAF in NSCLC cells with

kinase impaired BRAF, we knocked down CRAF expression using siRNA in NSCLC cells with kinase active or inactive BRAF. CRAF knockdown in H1666 (^{G466V}BRAF) and Cal12T (^{G466V}BRAF), but not H322 (^{wt}BRAF) or H661 (^{wt}BRAF) cells affected their viability as estimated using an MTT assay (Figure 4C), although the effects on Cal12T cells were modest. Given the limited number of cells lines expressing endogenous mutated BRAF, we utilized a less physiologic approach and transfected H661 cells with kinase active or inactive BRAF. Only cells expressing kinase-deficient BRAF showed reduced cell number after treatment with CRAF siRNA (Figure S10).

Dasatinib Induces RAF Dimerization

Consistent with the recent findings of Packer, et. al. we found that dasatinib induced RAF dimerization in cells with a *KRAS* mutation (A549) (10). In addition, dasatinib induced RAF dimerization in NSCLC cells with kinase impaired BRAF (Figure 5A). Although dasatinib caused a modest inhibition of RAF kinase activity, when corrected for the increase in total RAF dimers, there was no net inhibition of RAF in Cal12T and H1666 cells. Similarly, ERK was activated and p21 expression induced after dasatinib treatment in cells with kinase impaired BRAF consistent with oncogene induced senescence that is observed with *KRAS* or BRAF activation (Figure 2E) (11). ERK and MEK were inhibited in H661 and A549 cells, which is consistent with their lack of senescence following dasatinib exposure.

To further investigate the role of BRAF/CRAF heterodimerization in dasatinib-induced senescence, we transfected a BRAF mutant that prevents dimerization (^{R509H}BRAF) into NSCLC cells with endogenous ^{wt}BRAF either as a single mutation or in cis with ^{Y472C}BRAF (Figure 5B) (12). As before, the expression of ^{Y472C}BRAF led to the activation of ERK and increased sensitivity to dasatinib. The addition of ^{R509H}BRAF to ^{Y472C}BRAF inhibited ^{Y472C}BRAF's ability to activate ERK. NSCLC cells expressing the double mutations were not more sensitive to dasatinib than those expressing ^{wt}BRAF (Figure 5C).

Drug-induced RAF dimerization alone was not adequate to induce senescence in Cal12T or H1666 cells. Nilotinib induced more robust BRAF/CRAF dimerization than did dasatinib in Cal12T cells but did not induce senescence (Figure S11). Together these experiments demonstrate that dasatinib-induced RAF dimerization is essential, but not sufficient for dasatinib sensitivity.

A preliminary investigation of ERK-independent pathways downstream of RAF demonstrated no mutation-specific changes in BAD or JNK (13). Activated Aurora A and PLK1 were not detected by Western blotting (Figure S12) (14). Src family kinase inhibition with AZD0530 or knockdown with siRNA was not adequate to induce significant senescence or cytotoxicity (Figure S11 and S13).

The Combination of a BRAF Inhibitor and Dasatinib Leads to Synergistic Cytotoxicity in Cancer Cells Resistant to Dasatinib

The sensitivity of cancer cells with inactivating BRAF mutations to dasatinib suggests BRAF's synthetic lethality with a dasatinib target. We treated cell lines with ^{wt}BRAF and marked resistance to dasatinib (15, 16) with dasatinib plus the pan-RAF inhibitor sorafenib or dasatinib plus the BRAF inhibitor PLX4032 (vemurafenib) and then measured the treatment's effect on these lines' viability in vitro. In all cases, dasatinib enhanced the effect of the RAF inhibitors at clinically relevant doses (Figures 6 and S14) and formal analysis demonstrated synergy (Table S5). Although specific CRAF and pan-RAF inhibitors may be as effective as dasatinib in treating cancers with inactivating BRAF mutations, no credible

direct CRAF inhibitors are currently in clinical development, and pan-RAF inhibitors have poor in vivo activity and are not specific (17, 18).

Discussion

Metastatic NSCLC is a common and fatal disease with a 4-year survival rate of only 2%, but personalized therapies targeting specific genetic aberrations in NSCLC tumors are remarkably successful. We identified a patient with stage IV NSCLC with long term disease control by single-agent dasatinib therapy alone. His tumor harbored a kinase inactivating ^{Y472C}*BRAF* mutation that was likely responsible for its unusual sensitivity to dasatinib. Although effective treatments are available for melanoma patients with activating *BRAF* mutations, no such therapies are available for cancer patients who harbor inactivating mutations.

We did not observe any inactivating *BRAF* mutations in any other patients in that clinical trial, all of whom were nonresponders. As with previously characterized kinase inactivating *BRAF* mutations, ^{Y472C}*BRAF* expression led to CRAF, MEK, and ERK activation. Also, NSCLC cell lines with endogenous inactivating *BRAF* mutation underwent senescence when exposed to dasatinib; the cell lines' dasatinib sensitivity reversed with the overexpression of active BRAF. Whereas NSCLC cells transfected with an activating *BRAF* mutation were more resistant to dasatinib than were controls, transfection of NSCLC cells with kinase impaired *BRAF* led to their increased dasatinib sensitivity. These data and PX's pattern of clinical response are consistent with the conclusion that his tumor underwent senescence when exposed to dasatinib owing to its inactivating *BRAF* mutation. The mechanism of dasatinib-induced senescence and apoptosis in NSCLC cells expressing kinase impaired BRAF is unknown but may relate to increased RAF dimerization leading to ERK activation consistent with the paradigm of oncogene-induced senescence that occurs following moderate BRAF or KRAS activation (11). However, the modest effects on ERK suggest that ERK-independent pathways are involved as well.

BRAF mutations occur in only about 4% of NSCLC cases but are more common in other tumors, such as melanoma (50%) and papillary thyroid cancer (40%) (19, 20). The majority of BRAF mutations cause activation of the kinase (20). In NSCLC, 57% of mutations are ^{V600E}*BRAF* and the remaining 43% are a mixture of kinase inactivating, activating, and uncharacterized mutations (19). *CRAF* mutations are rare in all cancers (21).

Interactions between CRAF and BRAF are complex and incompletely understood despite several recent elegant studies in melanoma (4, 5, 22, 23). The pathway is not linear and is further complicated by the multiplicity of signaling molecule components; BRAF and CRAF are similar to each other but do not function identically and are not interchangeable and feedback pathways lead to inactivation of upstream components of the pathway (i.e., a negative feedback amplifier) (24). Additionally, MEK is not the only RAF substrate (25).

The complexity of BRAF-CRAF interactions is epitomized by the RAF inhibitor paradox. Specifically, inhibition of BRAF activity in cells that express ^{V600E}*BRAF* results in expected inhibition of ERK activity and subsequent apoptosis; melanomas with ^{V600E}*BRAF* respond clinically to BRAF inhibition (26). Paradoxically, BRAF inhibition in cells with active RAS leads to ERK activation via activation of CRAF (4). These observations are supported by previous research demonstrating that CRAF and BRAF heterodimers are more active than is either the CRAF or BRAF homodimer even when the BRAF protomer has an inactivating mutation (23).

These complex interactions of RAS, BRAF, and CRAF explain the existence and function of inactivating *BRAF* mutations in cancer cells, which superficially seem to be contrary to

natural selection. Activation of CRAF not only allows cells expressing kinase impaired BRAF to survive but also may further promote the cancer phenotype by activating non-ERK-dependent pathways or promoting aneuploidy (25, 27). CRAF activation may also be responsible for BRAF-inhibitor-induced squamous cell carcinomas (26).

Although much of the research involving BRAF has focused on melanoma, the BRAF pathway undoubtedly is active and important in NSCLC. RAS is commonly activated in NSCLC via upstream growth factor receptors or activating mutations. Active KRAS signals predominantly through CRAF, not BRAF (28). The role of CRAF in NSCLC has yet to be elucidated, although CRAF is overexpressed in NSCLC cells, and its forced overexpression leads to lung adenomas in a transgenic mouse model (29).

Oncogene-induced senescence occurs after activation of oncogenes such as RAS and RAF. Classically, it is mediated by activation of the p16^{INK4A}/Rb and/or p14^{ARF}/p53 tumor suppressor pathway. Senescence also can be induced and mediated by other pathways, such as those involving c-Src, STAT3, c-Myc, FOXO4, Chk2, and c-Jun-N-terminal-kinase. In the present study, dasatinib induced senescence in H1666 and Cal12T cells but induced apoptosis in H661 cells into which we transfected kinase impaired *BRAF* mutations. Possible reasons for this discrepancy are that the absolute level of endogenous ^{wt}BRAF, the level of mutant BRAF, or the wt:mutant BRAF ratio may influence the outcome of dasatinib-based treatment. The level of mutant BRAF expression was higher in the transfected cells, which also harbored a full complement of endogenous ^{wt}BRAF, than in non-transfected cells.

A limitation of our study was a paucity of cell lines and patients with endogenous inactivating *BRAF* mutations. Our conclusions would be greatly enhanced by demonstrating *BRAF* mutations in patients with other cancers that responded clinically to dasatinib. Another limitation of our study is that we did not fully elucidate the molecular mechanisms underlying dasatinib's biological effects in NSCLC cells with mutant BRAF.

Occasionally, spontaneous tumor regression occurs in melanoma and renal cell carcinoma cases and is thought to be immune mediated. Also, the activating ^{V600E}BRAF mutation in melanoma may induce an immune response. Although we cannot exclude such a possibility in the case of PX, spontaneous tumor regression is very rare in NSCLC cases. Instead, the dasatinib sensitivity of NSCLC cell lines with kinase impaired *BRAF* mutations is consistent with the patient having experienced a direct antitumor effect of dasatinib rather than an immune-mediated mechanism.

Other potential mechanisms that we could not fully explore are the roles of other dasatinib targets in mediating dasatinib sensitivity. Although no protein expression has been linked to dasatinib sensitivity, Sos et al. demonstrated that copy-number gains influence sensitivity of NSCLC cells to kinase inhibitors (7). H322 cells, which have c-Src amplification, are sensitive to dasatinib. PX's tumor had copy-number gains in several dasatinib targets. Because live tumor tissue specimens are not available from PX, determining whether his tumor was dependent upon HCK, DDR1, EPHA3, or ARG for survival is impossible (30).

In conclusion, we demonstrated that the kinase inactive mutation ^{Y472C}*BRAF* functions similarly to other known inactivating *BRAF* mutations and was likely responsible for PX's response to dasatinib. Moreover, the potential for synthetic lethality of combination therapy including dasatinib and BRAF inhibitors may lead to additional therapeutic options. Given that dasatinib and BRAF inhibitors are in clinical use, our work has potential for direct clinical applications.

Methods

Please see supplemental materials for: Immunofluorescence Microscopy, Copy-Number-Variation Analysis, Mutational Analysis, Cell Culture, Genomic DNA preparation, Immunohistochemistry, and Fluorescence In Situ Hybridization.

Materials

Antibodies used in this study were phosphorylated SFK (pSFK, Y416), pERK 1/2 (T202/Y204), p21^{cip}, Bcl2, total ERK, pMEK-1/2 (S217/221), phospho-EphA2 (Tyr594), and p-c-Kit (Y719) (Cell Signaling Technology); total CRAF (BD Biosciences); total BRAF, phospho-platelet-derived growth factor (pPDGFR ; Y754), total PDGFR , c-Kit, Flag M2, and agarose-conjugated CRAF (Santa Cruz Biotechnology); Flag and -actin (Sigma Chemical Co); and p53 (Dako). HP1- (Millipore); Anti mouse Alexa fluor 594 (Molecular Probes). Dasatinib, PLX4032 and sorafenib were purchased from Selleck Chemicals and prepared as 10 mM stock solutions in dimethyl sulfoxide.

Cytotoxicity Assay

Cytotoxicity in NSCLC cells was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described (31). Under each experimental condition, at least four independent wells were treated. Median effects of drugs on viability were calculated using the Chou-Talalay equation (32) with the CalcuSyn software program (Biosoft).

Senescence-Associated β -Galactosidase Staining

NSCLC cells were processed using a senescence-associated -galactosidase staining kit (Cell Signaling Technology) according to the manufacturer's instructions and visualized under an Olympus 1×71 phase microscope (Olympus America). In brief, upon completion of dasatinib-based treatment, cells were washed with phosphate-buffered saline to remove residual media and fixed. A -galactosidase staining solution containing X-gal was then added to the fixed cells and incubated at 37°C overnight in a dry incubator without CO₂. Fields with at least 100 cells were counted, in triplicate.

BRAF and CRAF Kinase Assays

The kinase activity of immunoprecipitated endogenous BRAF and CRAF protein from NSCLC cells; purified recombinant proteins; or immunoprecipitated Flag-tagged BRAF or Flag-tagged CRAF protein expressed in COS-7 cells was measured using an in vitro kinase assay (IVKA) kit for RAF (Millipore). Purified recombinant BRAF and CRAF (Sigma Chemical Co.) and immunoprecipitated proteins (technique described below using anti-Flag, -HA, -BRAF, or -CRAF antibodies) were incubated with 100–250 μ M ATP/Mg²⁺ along with 1 μ g of inactive recombinant GST-MEK-1 at 30°C for 30 min and subsequently boiled with 1x sample buffer to stop the reaction. MEK-1 activation was quantified by measuring the phospho-specific MEK-1 band (ImageJ software program; National Institutes of Health) after Western blotting (described below).

Cell-Cycle, Proliferation and Apoptosis Assays

For cell-cycle analysis, cells were harvested, fixed, and stained with propidium iodide, and their DNA content was analyzed using a cytofluorimeter and fluorescence-activated cell sorter (FACS) (FACScan; Becton Dickinson) and using the ModFit software program (Verity Software House) (31). To measure apoptosis, fixed cells were subjected to terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) staining according to the manufacturer's procedure (APO-BRDU kit; Phoenix Flow Systems) and quantitated using the FACS (31). BrdU incorporation was measured according to the manufacturer's

instruction (BrdU Flow Kits, BD Biosciences). Briefly, subconfluent cell cultures were treated with DMSO or 150 nM dasatinib for 72 hours, then labeled with 10 μ M BrdU for 4 hr. Cells were trypsinized, fixed, and stained with FITC-conjugated anti-BrdU antibody and 7-Aminoactinomycin D (7-AAD). Samples were analyzed by two-dimensional flow cytometry to detect both fluorescein and 7-AAD.

Transfection of NSCLC Cells with a Small Interfering RNA

Cells were harvested, washed, and suspended (10^6 cells/100 μ L) in Nucleofector Solution V (Amaxa), and small interfering RNA (siRNA; 200 pmol/100 μ L) was added to the cells. Cells were then electroporated using the Nucleofector program U24 (Amaxa) and diluted with a prewarmed 500- μ L RPMI medium supplemented with 10% serum and plated onto 60-mm plates and the medium was changed 16 h later. The c-Src and CRAF siRNAs were predesigned as sets of four independent sequences (siGENOME SMARTpool; Dharmacon). Controls were cells transfected with a nontargeting (scrambled) siRNA and mock-transfected cells (i.e., no siRNA).

Site-Directed Mutagenesis

Flag-tagged *wtBRAF* and *V600EBRAF* plasmids were provided by Dr. Walter Kolch (Systems Biology Ireland and The Conway Institute, University College Dublin). The *wtBRAF* construct was used as a template to create the *Y472CBRAF*, *G466VBRAF*, *R509HBRAF* and *Y472C/R509HBRAF* mutations using the QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The sense mutagenic primers used were 5' - GATCATTGGAACAGTCTGCAAGGGAAAGTGGCATGGT -3' for *Y472CBRAF* and 5' - GTGGGACAAAGAATTGGATCTGTATCATTGGAACA GTC -3' for *G466VBRAF*, 5' - GTAGGAGTACTCAGGAAAACACACCATGTGAATATCCTACTCT -3' for *R509HBRAF* and *Y472C/R509HBRAF*. To confirm successful introduction of the mutations, six different plasmids for each mutant were Sanger sequenced.

Immunoprecipitation and Western Blot Analysis

For both Western blot and immunoprecipitation (IP) analysis, cells were lysed on ice, and the lysates were centrifuged at 20,000g for 5 min at 4°C as described previously (31). For IP, equal amounts of the protein cell lysate supernatant (500 mg) were precleared with protein A and G sepharose beads (Invitrogen). The precleared lysate was incubated with an IP antibody (anti-Flag or anti-CRAF) overnight. The immunoprecipitates were washed four times with an immunocomplex wash buffer (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 1% Triton X-100, 1 mmol/L egtazic acid, 1 mmol/L ethylenediaminetetraacetic acid, 1% glycerol, 20 μ g/mL leupeptin, 10 μ g/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium vanadate) and boiled with 1 $\times$ sample buffer for 5 min. For both the IP and Western blot analysis, equal protein aliquots were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, immunoblotted with a primary antibody, and detected using a horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) and ECL reagent (Amersham Biosciences).

Clinical Tissue Specimens

Patients' tissue specimens were obtained in an MD Anderson Institutional Review Board-approved clinical trial in which all participants gave permission for testing of residual tumor tissue. For PX, residual tissue from a metastatic axillary lymph node resected 2 months before treatment was used. The metastasis was formalin-fixed and paraffin-embedded (FFPE) according to a standard protocol. One 4- μ m tissue section was cut and stained with

hematoxylin and eosin. Histopathological tumor examination and visual estimation of the tumor area and tumor-cell percentage were conducted by a pathologist. The tissue section had a 2.5×1.8 cm lymph node extensively infiltrated with malignant epithelial cells representing ~60 % of the whole tissue section. Five 10- μ m unstained tissue sections were cut and placed in sterile tubes for genomic DNA (gDNA) extraction and isolation.

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Summary

Induction of tumor cell senescence may explain the response of a patient with BRAF-kinase impaired lung cancer to the multi-kinase inhibitor dasatinib.

Figure 1. $Y472C$ *BRAF* is biochemically similar to $G466V$ *BRAF* but different from $V600E$ *BRAF*. **A**) Kinase activity in Flag-tagged BRAF proteins with the noted mutations. After immunoprecipitation (IP) from COS7 cells and incubation with kinase-dead MEK (substrate) and ATP, MEK phosphorylation was measured using Western blotting and quantitated with ImageJ. **(B–D)** The effect of the expression of mutated BRAF proteins on MEK and ERK activity in intact cells. Western blotting was performed in **(B)** COS7, **(C)** H661, and **(D)** H226 cells transfected with *BRAF* constructs. **E**) The activity of CRAF in H661 cells transfected with mutated BRAF, as measured by incubating immunoprecipitated CRAF with kinase-dead MEK (substrate) and ATP. **F**) CRAF kinase activity and BRAF-CRAF binding, as measured in COS7 cells transfected with FLAG-tagged BRAF with the indicated mutations by kinase activity (IVKA) or IP of CRAF followed by blotting with anti-Flag and anti-BRAF antibodies (binding). WT, wild-type.

Figure 2.

NSCLC cells with an inactivating BRAF mutation undergo senescence when exposed to dasatinib. **A–D**) Biological effects of dasatinib in NSCLC cells with *BRAF* mutations. NSCLC cells were incubated with 150 nM dasatinib or a control vehicle for 72 h followed by β -galactosidase (**A**) staining and (**B**) quantification, (**C**) cell cycle analysis using propidium iodide staining and FACS analysis, or (**D**) BrdU incorporation. **E**) The effect of dasatinib on downstream signaling as measured by Western blotting of NSCLC cells incubated with 150nM dasatinib for 24 h. (**F**) The reversibility of dasatinib-induced senescence. Cal12T cells were incubated with 150nM dasatinib or fresh medium (vehicle,

but no drug) for the indicated times (left panel) and senescence was measured with - galactosidase staining using light microscopy. * $P < 0.05$. ** $P < 0.001$.

Figure 3.

Expression of kinase inactive and active BRAF leads to sensitivity and resistance to dasatinib, respectively, in NSCLC cells. (**A, B, D**) Cell viability was assayed following dasatinib treatment in NSCLC cells transfected with *BRAF* constructs. Following transfection with the noted *BRAF* constructs (**A**) H661, (**B**) H226, and (**D**) H1666 cells were and incubated with dasatinib at increasing doses for 72 h and cell viability was estimated using the MTT assay. The half-maximal inhibitory concentration (IC₅₀) of dasatinib was calculated for H661. (**C**) Apoptosis in H661 cells expressing BRAF with the noted mutations as measured using the terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling assay.

Figure 4.

Dasatinib indirectly inhibits CRAF activity in cells with an inactivating *BRAF* mutation but not in cells with *wt* *BRAF* or *V600E* *BRAF*. **A**) Kinase activity of isolated BRAF and CRAF incubated with dasatinib. Purified recombinant BRAF and CRAF were incubated with 100 nM dasatinib in the presence of inactive MEK-1 and ATP. Kinase activity was measured by phosphorylation of MEK-1. **B**) The effect of dasatinib on the kinase activity of CRAF in intact cells expressing various BRAF constructs. COS-7 cells were transfected with Flag-tagged CRAF along with HA-tagged BRAF constructs with the noted mutations 24 h prior to dasatinib treatment. Transfected cells were incubated with 150 nM dasatinib or vehicle for 24 h followed by immunoprecipitation with an anti-Flag antibody. The resulting lysate was subjected to a Western blot with anti-Flag and anti-HA antibodies (lower two rows) and CRAF kinase activity was measured by incubating Flag-immunoprecipitated CRAF with kinase-dead MEK-1 (substrate) and ATP (IVKA, top row). MEK activation was quantitated and normalized with total FLAG. **(C)** The effect of CRAF knock down (KD) on NSCLC cell number in cells with endogenous *BRAF* mutations. Untransfected Cat12T, H1666, H322, and H661 cells were incubated with CRAF siRNA, and their viability was estimated using an MTT assay. In all cases, Western blotting was used to confirm CRAF knockdown. At 120h Cal12T cells lost dasatinib sensitivity and CRAF expression recovered.

Figure 5. Dasatinib induces RAF dimerization in NSCLC cells which is necessary for sensitivity in cells expressing kinase impaired BRAF. (A) Dasatinib induced RAF dimerization. NSCLC cells were incubated with 150 nM dasatinib for 72h then CRAF was immunoprecipitated. The resulting lysate was split with part used for blotting with antibodies to BRAF or CRAF (to measure dimerization) and part used to measure CRAF kinase activity using MEK as a substrate. (B–C) Introduction of a mutation that interferes with RAF dimerization abrogates the effects of kinase deficient BRAF on dasatinib sensitivity. H661 cells transfected with the BRAF constructs as indicated were subjected to Western blotting (B) and an MTT assay (C).

Figure 6.

Dasatinib enhances the cytotoxicity of PLX4032 in cancer cells resistant to dasatinib. Dasatinib-resistant NSCLC cells were incubated with single agent dasatinib (50–600 nM) or single agent PLX5032 (1000–12000 nM) or in combination of both (dasatinib:PLX4032 = 1:20) for 72 h, and their viability was estimated using an MTT assay.

Table 1

Lung cancer cell sensitivity to dasatinib

Cell line	BRAF	BRAF mutation type	Dasatinib IC ₅₀ (nM)	Kinase activity (fold wt) [*]	Other known mutations or polymorphisms
H1666	G466V	Inactivating	158	0-65	CDKN2A, STK11
H322	wt	wt	142	1	TP53, CDKN2A, STK11
Cal-12T	G466V	Inactivating	238	0-65	TP53, CDKN2A
H661	wt	wt	1484	1	TP53, CDKN2A, SMARCA4
H460	wt	wt	>2500	1	KRAS, CDKN2A, STK11, PIK3CA
H1568	wt	wt	3321	1	None known
H226	wt	wt	>5000	1	TP53
A549	wt	wt	>5000	1	KRAS, CDKN2A, SMARCA4, STK11
H522	wt	wt	>5000	1	TP53
H2087	L597V	Activating	>5000	64	NRAS, TP53, DGKB, DGKB, PTPRT, ATM, BAI3, HECW1, HEPH, NLRP4, NTRK3, PDZRN4, PTPRM
H1755	G469A	Activating	>5000	266	KRAS, TP53
H1395	G469A	Activating	>5000	266	STK11, NLRP1, RPTPT, ATM, BIRC6, NLRP4, TRIM36
HCC364	V600E	Activating	>5000	478	None known
H2405	In-frame deletion	Unknown	>8000	ND	TP53

^{*} Based on published values determined using an in vitro kinase assay (33, 34).
 ND, not done