Sorafenib Inhibits Non–Small Cell Lung Cancer Cell Growth by Targeting B-RAF in *KRAS* Wild-Type Cells and C-RAF in *KRAS* Mutant Cells

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Abstract

Sorafenib is a multikinase inhibitor whose targets include B-RAF and C-RAF, both of which function in the extracellular signal-regulated kinase (ERK) signaling pathway but which also have distinct downstream targets. The relative effects of sorafenib on B-RAF and C-RAF signaling in tumor cells remain unclear, however. We have now examined the effects of sorafenib as well as of B-RAF or C-RAF depletion by RNA interference on cell growth and ERK signaling in non–small cell lung cancer (NSCLC) cell lines with or without *KRAS* mutations. Sorafenib inhibited ERK phosphorylation in cells with wild-type *KRAS* but not in those with mutant *KRAS*. Despite this difference, sorafenib inhibited cell growth and induced G_1 arrest in both cell types. Depletion of B-RAF, but not that of C-RAF, inhibited ERK phosphorylation as well as suppressed cell growth and induced G_1 arrest in cells with wild-type *KRAS*. In contrast, depletion of C-RAF inhibited cell growth and induced G_1 arrest, without affecting ERK phosphorylation, in cells with mutant *KRAS*; depletion of B-RAF did not induce G_1 arrest in these cells. These data suggest that B-RAF-ERK signaling and C-RAF signaling play the dominant roles in regulation of cell growth in NSCLC cells with wild-type or mutant *KRAS*, respectively. The G_1 arrest induced by either C-RAF depletion or sorafenib in cells with mutant *KRAS* was associated with down-regulation of cyclin E. Our results thus suggest that sorafenib inhibits NSCLC cell growth by targeting B-RAF in cells with wild-type *KRAS* and C-RAF in those with mutant *KRAS*. [Cancer Res 2009;69(16):6515–21]

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (1). Treatment options are limited for patients with advanced metastatic lung cancer, with traditional cytotoxic chemotherapy conferring only a limited survival benefit. Target-based therapies are therefore being pursued as potential treatment alternatives. The RAS-RAF-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase-ERK signaling pathway is a promising therapeutic target given its central role in regulation of mammalian cell proliferation, relaying extracellular signals from ligand-bound receptor tyrosine kinases (RTK) at the cell surface to the nucleus via a cascade of specific phosphorylation events and beginning with the activation of the small GTPase RAS (2). Much attention is thus being focused on the development of inhibitors of this pathway.

RAF was the first effector kinase downstream of RAS to be identified (3). To date, the most successful clinical inhibitor of RAF activity is sorafenib (Nexavar, BAY 43-9006), an orally available compound that has received approval by the U.S. Food and Drug Administration for the treatment of advanced renal cell carcinoma and hepatocellular carcinoma. Sorafenib is also currently undergoing clinical evaluation for a variety of additional cancers, including non–small cell lung cancer (NSCLC; refs. 4–7).

The mutational status of *RAS* and *B-RAF* genes is thought to affect the sensitivity of tumor cell lines to sorafenib as a result of the inappropriate activation by such mutations of the MAPK pathway mediated by ERK (8, 9). The sensitivity of tumor cell lines with different *RAS* mutations to sorafenib is less well characterized than is that of those with *B-RAF* mutations (10–14). Despite promising results of clinical trials of sorafenib monotherapy in NSCLC patients (4–7), little is known of the possible differences in the sorafenib sensitivity of NSCLC cells according to the mutational status of *KRAS*. We have therefore now examined the effects of RAF inhibition on the growth of NSCLC cells with or without *KRAS* mutations and further investigated the mechanisms of such effects.

Materials and Methods

Cell culture and reagents. The human NSCLC cell lines NCI-H292 (H292), LK-2, Sq-1, NCI-H520 (H520), PC9, NCI-H1650 (H1650), HCC827, NCI-H1975 (H1975), A549, NCI-H460 (H460), NCI-H23 (H23), NCI-H358 (H358), and NCI-H1299 (H1299) were obtained from the American Type Culture Collection. Ma70 cells were obtained as previously described (15). All cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. Sorafenib was kindly provided by Bayer Pharmaceutical, dissolved in DMSO, and stored in aliquots at −20°C.

Assay of anchorage-dependent cell growth [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay]. Cells were plated in 96-well flat-bottomed plates and cultured for 24 h before exposure to various concentrations of sorafenib for 72 h. TetraColor One (5 mmol/L tetrazolium monosodium salt and 0.2 mmol/L 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems). Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of sorafenib resulting in 50% growth inhibition (IC_{50}) was calculated.

Assay of anchorage-independent colony formation in soft agar. Anchorage-independent cell proliferation in soft agar was assayed with the use of a CytoSelect 96-Well Cell Transformation Assay (Cell Biolabs). In brief, cells were cultured for 7 d in complete medium containing soft agar and various concentrations of sorafenib. The agar matrix was then solubilized, the cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

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bromide (MTT) and lysed, and the absorbance at 570 nm was measured relative to that at a reference wavelength of 690 nm. Normalized absorbance values were expressed as a percentage of that for untreated cells, and the IC_{50} of sorafenib for inhibition of colony formation was calculated.

Cell cycle analysis. Cells were harvested, washed with PBS, fixed with 70% methanol, washed again with PBS, and stained with propidium iodide (0.05 mg/mL) in a solution containing 0.1% Triton X-100, 0.1 mmol/L EDTA, and RNase A (0.05 mg/mL). The stained cells ($\sim 1 \times 10^6$) were then analyzed for DNA content with a flow cytometer (FACSCalibur, Becton Dickinson) and ModFit software (Verity Software House).

Immunoblot analysis. Cells were washed twice with ice-cold PBS and then lysed in a solution containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin. The protein concentration of lysates was determined with the Bradford reagent (Bio-Rad), and equal amounts of protein were subjected to SDS-PAGE on a 7.5% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in PBS for 1 h at room temperature before incubation overnight at 4°C with rabbit polyclonal antibodies to human phosphorylated ERK (1:1,000 dilution; Santa Cruz Biotechnology), ERK (1:1,000 dilution; Santa Cruz Biotechnology), FLAG epitope (1:1,000 dilution; Cell Signaling Technology), B-RAF (1:1,000 dilution; Santa Cruz Biotechnology), C-RAF (1:1,000 dilution; Cell Signaling Technology), or β-actin (1:500 dilution; Sigma) or with mouse monoclonal antibodies to cyclin E (1:1,000 dilution; Santa Cruz Biotechnology). The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase–conjugated goat antibodies to rabbit (Sigma) or mouse (Santa Cruz Biotechnology) immunoglobulin G. Immune complexes were finally detected with chemiluminescence reagents (Perkin-Elmer Life Science).

Forced expression of KRAS-V12. An expression vector for FLAG-tagged human KRAS-V12 was constructed by inserting the corresponding cDNA into the pcDNA3 plasmid (Invitrogen). The expression vector was introduced into H1299 cells by transfection for 48 h with the use of the Lipofectamine 2000 reagent (Invitrogen).

Gene silencing. Cells were plated at 50% to 60% confluence in six-well plates or 25-cm^2 flasks and then incubated for 24 h before transient transfection for 48 h with small interfering RNAs (siRNAs) mixed with the Lipofectamine reagent. The siRNAs specific for B-RAF (5′-AGACAGGAAUCGAAUGAAA-3′) or C-RAF (5′-CCUCACGCCUUCACCUUUA-3′) mRNAs were obtained from Dharmacon, and a nonspecific siRNA (control) was obtained from Nippon EGT. The cells were then subjected to immunoblot analysis or flow cytometry.

Statistical analysis. Data were analyzed by Student's two-tailed *t* test. A *P* value of <0.05 was considered statistically significant.

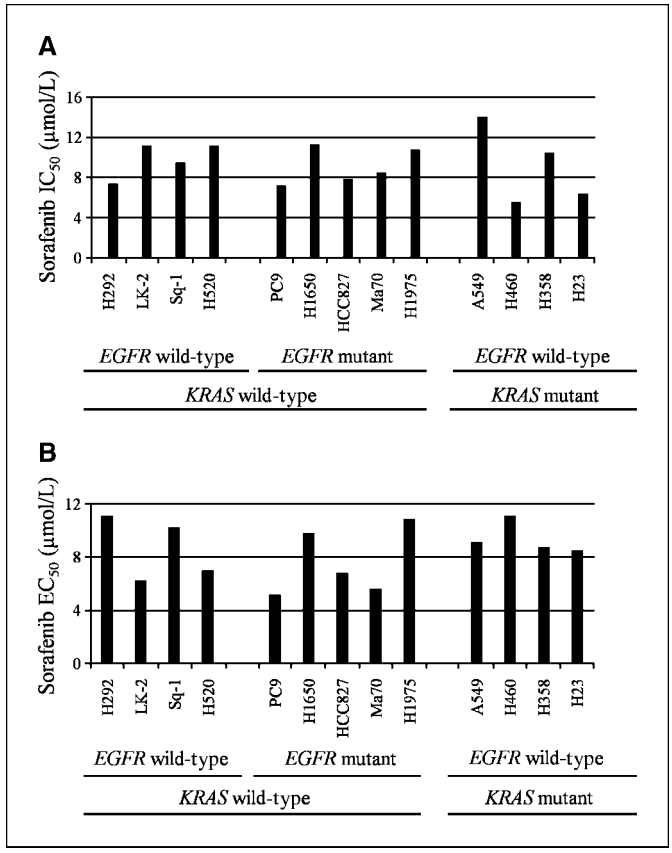

Figure 1. Effects of sorafenib on the growth of NSCLC cell lines classified according to *KRAS* and *EGFR* mutational status. *A,* the indicated NSCLC cell lines were cultured for 72 h in complete culture medium containing various concentrations of sorafenib, after which cell viability was assessed with the MTT assay and the IC_{50} values of sorafenib for inhibition of cell growth were determined. *B,* the indicated NSCLC cell lines were cultured for 7 d in complete medium containing soft agar and various concentrations of sorafenib, after which colony formation was evaluated and the IC_{50} values of sorafenib for inhibition of anchorage-independent cell proliferation were determined. All data are means of triplicates from representative experiments that were repeated on three separate occasions.

Results

Sorafenib inhibits cell growth by inducing G_1 arrest in NSCLC cell lines independently of *KRAS* genotype. The various isoforms of RAF are the principal effectors of RAS in the ERK signaling pathway, and mutant RAS proteins trigger persistent activation of downstream effectors (3). To determine whether the mutational status of *KRAS* might affect the sensitivity of NSCLC cells to sorafenib, an inhibitor of the kinase activity of RAF (16), we first examined the effects of this drug on the anchorage-dependent growth of NSCLC cells with or without *KRAS* mutations by the MTT assay. Sorafenib inhibited cell growth with IC_{50} values ranging from 7.4 to 11.3 μmol/L in NSCLC cells with wild-type *KRAS* and from 5.6 to 14.1 μmol/L in those with mutant *KRAS* (Fig. 1*A*), values that are within the clinically relevant concentration range for this drug (17). This inhibitory effect of sorafenib in cells with wild-type *KRAS* also seemed to be independent of whether the cells contained a mutant version of the *epidermal growth factor receptor* (*EGFR*) gene. We next investigated the effects of sorafenib on anchorage-independent colony formation in soft agar, a more clinically relevant model of NSCLC cell proliferation. Sorafenib inhibited anchorage-independent colony formation with IC_{50} values of 5.6 to 11.1 μmol/L in cells with wild-type *KRAS* and of 8.5 to 11.1 μmol/L in those with mutant *KRAS* (Fig. 1*B*). These data thus indicated that sorafenib inhibits the growth of NSCLC cells in a manner independent of *KRAS* mutational status.

To investigate the mechanism by which sorafenib inhibits NSCLC cell growth, we examined the cell cycle profile by flow cytometry. Sorafenib increased the proportion of cells in G_1 phase of the cell cycle and reduced that of cells in S or G_2-M phases in all tested cell lines regardless of *KRAS* mutational status (Fig. 2). Sorafenib did not increase the proportion of cells in sub-G_1 phase, a characteristic of apoptosis. These data thus indicated that sorafenib inhibits cell growth by inducing arrest of the cell cycle in G_1 phase.

Effects of sorafenib on the ERK signaling pathway in NSCLC cell lines. To examine the effects of sorafenib on the ERK signaling pathway in NSCLC cells, we performed immunoblot analysis with antibodies specific for phosphorylated (activated) ERK. Sorafenib markedly inhibited ERK phosphorylation in cells with wild-type *KRAS* regardless of the mutational status of *EGFR* (Fig. 3*A*). In contrast, sorafenib had no effect on the level of ERK phosphorylation in cells

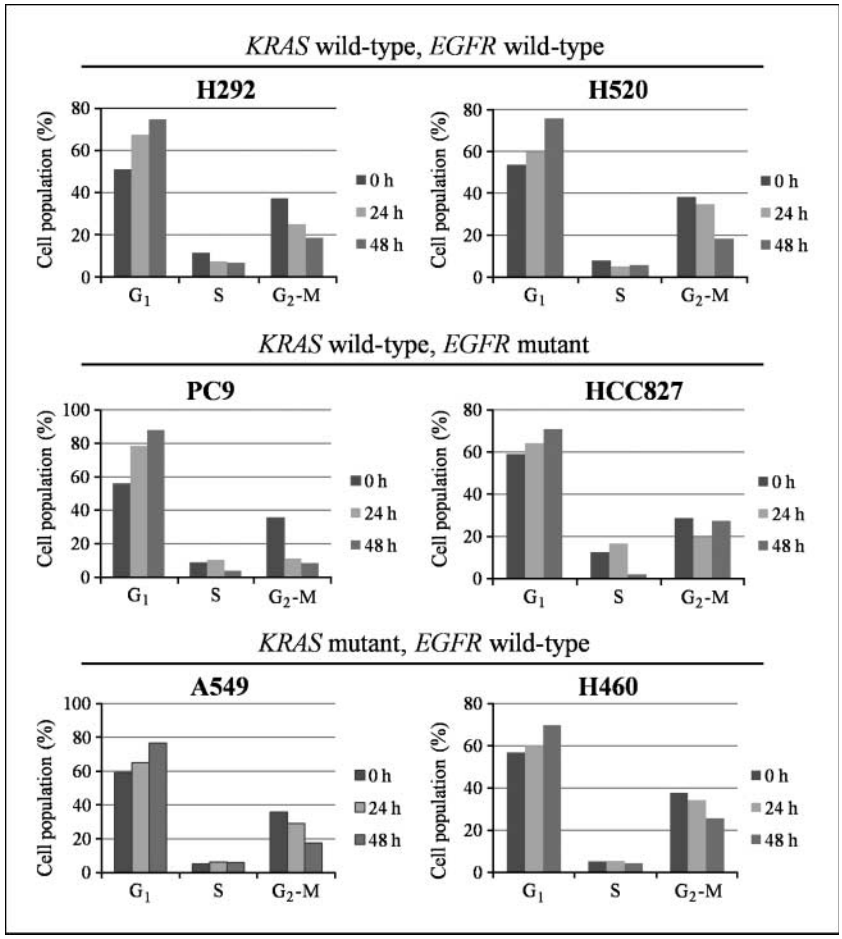

Figure 2. Effects of sorafenib on cell cycle distribution in NSCLC cells classified according to *KRAS* and *EGFR* status. Cells were incubated for 0, 24, or 48 h in complete culture medium containing 15 μmol/L sorafenib and were then fixed, stained with propidium iodide, and analyzed for cell cycle distribution by flow cytometry. All data are means of triplicates from representative experiments that were repeated on three separate occasions.

with mutant *KRAS*. To investigate further whether the effect of sorafenib on ERK phosphorylation is dependent on *KRAS* mutational status, we introduced an expression vector for FLAG epitope-tagged KRAS with the activating Val^{12} mutation (KRAS-V12) into the human NSCLC cell line H1299, which harbors wild-type endogenous *KRAS*. Whereas sorafenib inhibited ERK phosphorylation in nontransfected cells or cells transfected with the empty vector, it failed to do so in cells expressing KRAS-V12 (Fig. 3*B*). These results thus suggested that sorafenib blocks the ERK signaling pathway only in NSCLC cells harboring wild-type *KRAS*.

B-RAF but not C-RAF depletion inhibits ERK phosphorylation in NSCLC cells with wild-type or mutant KRAS. The mammalian RAF family includes A-RAF, B-RAF, and C-RAF, all of which function in the ERK pathway but also have different downstream phosphorylation targets and play distinct roles in signaling (18). Although suggested to be a B-RAF inhibitor, sorafenib inhibits the activity of C-RAF with a potency 4-fold that apparent for B-RAF (16). To investigate the downstream consequences of B-RAF and C-RAF signaling in NSCLC cells, we examined the effects of the depletion of these kinases with a siRNA-based approach. Immunoblot analysis revealed

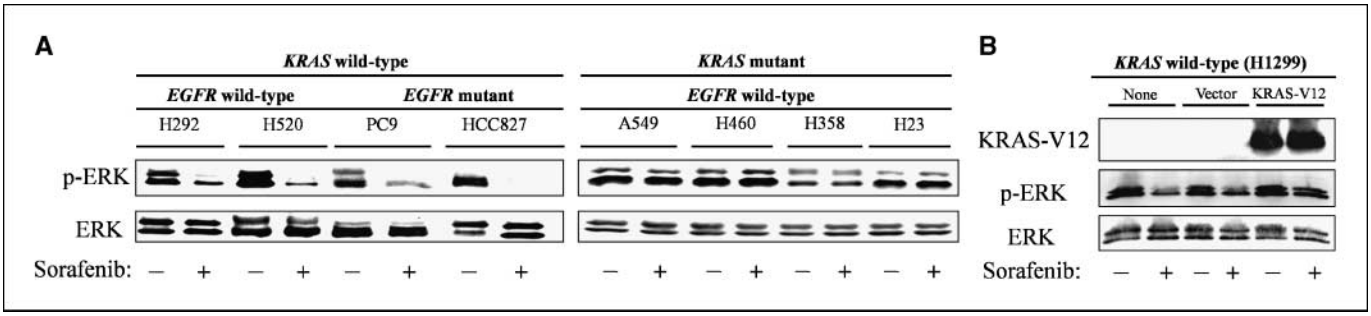

Figure 3. Effects of sorafenib on ERK phosphorylation in NSCLC cells classified according to *KRAS* and *EGFR* status. *A,* cells were incubated for 2 h in the presence or absence of sorafenib (15 μmol/L), after which cell lysates (25 μg of soluble protein) were subjected to immunoblot analysis with antibodies to phosphorylated (*p-ERK*) or total forms of ERK. *B,* H1299 cells were transiently transfected (or not) for 48 h with an expression vector for FLAG-tagged KRAS-V12 or with the corresponding empty vector and were then incubated for 2 h in the presence or absence of sorafenib (15 μmol/L). Cell lysates (25 μg of soluble protein) were then subjected to immunoblot analysis with antibodies to FLAG and to phosphorylated or total forms of ERK.

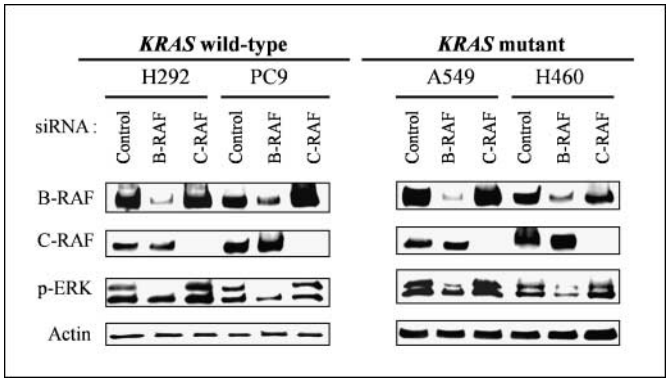

Figure 4. Effects of transient depletion of B-RAF or C-RAF on ERK phosphorylation in NSCLC cells. Cells harboring wild-type or mutant *KRAS* were transfected with nonspecific (control), B-RAF, or C-RAF siRNAs for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to B-RAF, C-RAF, phosphorylated ERK, and β-actin (loading control).

that transfection of NSCLC cells with siRNAs specific for B-RAF or C-RAF mRNAs resulted in marked and selective depletion of the corresponding protein (Fig. 4). Such depletion of B-RAF resulted in inhibition of ERK phosphorylation in cells harboring wild-type or mutant *KRAS*, whereas depletion of C-RAF had no such effect (Fig. 4). These data thus suggested that depletion of B-RAF, but not that of C-RAF, inhibits ERK phosphorylation regardless of *KRAS* status.

Effects of RAF depletion on NSCLC cell proliferation. We next examined the effects of B-RAF or C-RAF depletion on NSCLC cell proliferation and cell cycle distribution. Depletion of B-RAF resulted in significant inhibition of cell proliferation (Fig. 5*A*) and an increase in the proportion of cells in G_1 phase of the cell cycle (Fig. 5*B*), whereas depletion of C-RAF had no such effects, in NSCLC cells harboring wild-type *KRAS*. In contrast, depletion of C-RAF induced significant inhibition of cell proliferation (Fig. 5*A*) and an increase in the proportion of cells in G_1 phase (Fig. 5*B*), whereas depletion of B-RAF had only a less pronounced effect on cell proliferation, in NSCLC cells with mutant *KRAS*. These data thus suggested that B-RAF-ERK signaling regulates cell proliferation in NSCLC cells with wild-type *KRAS*, whereas C-RAF signaling mediates such regulation in NSCLC cells with mutant *KRAS*.

Sorafenib or C-RAF depletion inhibits cyclin E expression in NSCLC cells with mutant *KRAS*. Finally, to characterize further the growth inhibition and G_1 arrest induced by C-RAF depletion or sorafenib in NSCLC cells with mutant *KRAS*, we examined the expression of cyclin E, an essential promoter of the transition from G_1 to S phase of the cell cycle (19). Immunoblot analysis revealed that depletion of C-RAF in A549 or H460 cells resulted in pronounced inhibition of cyclin E expression, whereas depletion of B-RAF had no such effect (Fig. 6). Exposure of the cells to sorafenib also induced loss of cyclin E (Fig. 6). These results thus suggest that the G_1 arrest induced by depletion of C-RAF or by sorafenib in NSCLC cells with mutant *KRAS* may be attributable to the down-regulation of cyclin E.

Discussion

RAS is an upstream component of the ERK signaling pathway, which is aberrantly activated by oncogenic mutations of RAS genes. Among RAS family genes, mutations of *KRAS* are most common in solid malignancies, including NSCLC (8, 20, 21). Indeed, *KRAS* mutations have been associated with poor prognosis and resistance to conventional cytotoxic chemotherapy in NSCLC (22–24). Whereas EGFR tyrosine kinase inhibitors are most efficacious in NSCLC patients with *EGFR* mutations, *KRAS* mutations are associated with resistance to these agents (25–28). The development of therapeutic strategies for NSCLC patients with *KRAS* mutations is thus an important clinical goal. RAF serine-threonine kinases are the principal effectors of RAS in the ERK signaling pathway. Given the key role of this pathway in tumor growth, RAF is a potential target for cancer therapy.

Sorafenib is an orally available compound that has been developed as a multikinase inhibitor with activity against RAF and several RTKs. The sensitivity of cancer cells to sorafenib might be expected to be affected by *KRAS* status, given that *KRAS* mutations result in activation of the ERK pathway (8). However, as far as we are aware, no previous study has compared sorafenib sensitivity among a panel of tumor cell lines of different *KRAS* mutational status. We have now evaluated the effects of sorafenib on the growth of NSCLC cells harboring wild-type or mutant forms of *KRAS* with two different assay systems, the MTT assay and anchorage-independent colony formation assay, given that previous studies have revealed differences in the sensitivity of cells to tested drugs between these two assay systems (29). The IC_{50} values for inhibition of cell growth by sorafenib in these assays have generally been found to be well below 15 μmol/L, the maximum achievable plasma concentration of this drug (17). We found that the potency of sorafenib for inhibition of cell growth was similar for NSCLC cells regardless of *KRAS* mutational status in both assay systems. We also performed a longer-term clonogenic survival assay and again found that sorafenib inhibited the survival of NSCLC cells regardless of *KRAS* status (data not shown). These results thus indicate that sorafenib inhibits the growth of NSCLC cells with mutant *KRAS* as well as it does that of those with wild-type *KRAS* in a clinically relevant concentration range.

We have shown that sorafenib inhibited ERK phosphorylation and induced G_1 arrest in NSCLC cells with wild-type *KRAS*, consistent with previous results obtained with several cancer cell lines harboring wild-type *KRAS* (13, 30, 31). Inhibition of the ERK signaling pathway, as reflected by a reduced level of ERK phosphorylation, results in inhibition of cell proliferation and induction of G_1 arrest in various cell types (32–35). In the present study, we found that depletion of B-RAF by RNA interference also inhibited ERK phosphorylation as well as attenuated cell proliferation and induced G_1 arrest in NSCLC cells with wild-type *KRAS*. These results suggest that inhibition of B-RAF-ERK signaling contributes to suppression of the growth of NSCLC cells harboring wild-type *KRAS* by sorafenib. Consistent with these findings, the specific B-RAF inhibitor SB-590885 was previously shown to inhibit ERK phosphorylation and to induce G_1 arrest in melanoma cells with wild-type *KRAS* (36, 37). In contrast, we found that depletion of C-RAF did not result in inhibition of ERK phosphorylation in NSCLC cells. ERK activation was previously shown to be conserved in cells derived from C-RAF knockout mice, suggesting that C-RAF is dispensable for ERK signaling (38, 39). Together, the present data suggest that B-RAF-ERK signaling, rather than C-RAF signaling, is a potential therapeutic target in NSCLC cells with wild-type *KRAS*.

We showed that ERK phosphorylation was not inhibited by sorafenib in two NSCLC cell lines (A549 and H460) harboring mutant *KRAS*, consistent with previous observations (16). We further showed this to be the case in two additional such cell lines (H358 and H23). Such results were previously suggested to be due to the existence of RAF-independent ERK activation in

NSCLC cells with mutant *KRAS* (16). However, we have now shown that B-RAF depletion resulted in inhibition of ERK activation in these cells. Our data therefore suggest that sorafenib is not able to attenuate the constitutive activation of the B-RAF-ERK pathway characteristic of NSCLC cells harboring mutant *KRAS* (40). Despite the sustained activation of B-RAF-ERK signaling in such cells, sorafenib inhibited cell proliferation and induced G_1 arrest in NSCLC cells with mutant *KRAS* as well as in those with wild-type *KRAS*. These data suggest that sorafenib targets a different pathway in its inhibitory effect on cell growth in NSCLC cells with mutant *KRAS*. Whereas sorafenib inhibits the kinase activity of both B-RAF and C-RAF, it shows a higher affinity for C-RAF (16). We found that depletion of C-RAF by RNA interference inhibited cell proliferation and induced G_1 arrest, without affecting ERK phosphorylation, in NSCLC cells with mutant *KRAS*, whereas it did not exhibit such effects in NSCLC cells harboring wild-type *KRAS*. Depletion of B-RAF also inhibited the growth of NSCLC cells with mutant *KRAS*, although this effect was not as pronounced as that in those with wild-type *KRAS*. These data indicate that NSCLC cells with mutant *KRAS* are dependent on C-RAF signaling to a greater extent than on B-RAF-ERK signaling for cell proliferation but that both pathways participate in regulation of the growth of these cells. Melanoma cells that have acquired resistance to a specific B-RAF inhibitor were recently shown to have switched their dependency from B-RAF to C-RAF (41). These observations suggest that RAF proteins are functionally interchangeable in the regulation of cell growth. Our data thus indicate that C-RAF signaling is a potential therapeutic target in NSCLC cells with mutant *KRAS*. RAF family proteins are also implicated in regulation of cell cycle progression in a manner independent of the ERK pathway (18, 38, 42, 43). C-RAF has been shown to exist in a complex with Cdc25, which activates the cyclin E-Cdk2 complex and promotes the G_1-S phase transition (44, 45). Cyclin E is thus postulated to be a downstream effector of C-RAF. In the present study, we found that either C-RAF depletion or sorafenib treatment induced G_1 arrest and down-regulation of cyclin E in NSCLC cells with mutant *KRAS*. Although we cannot exclude a possible role for other cell cycle

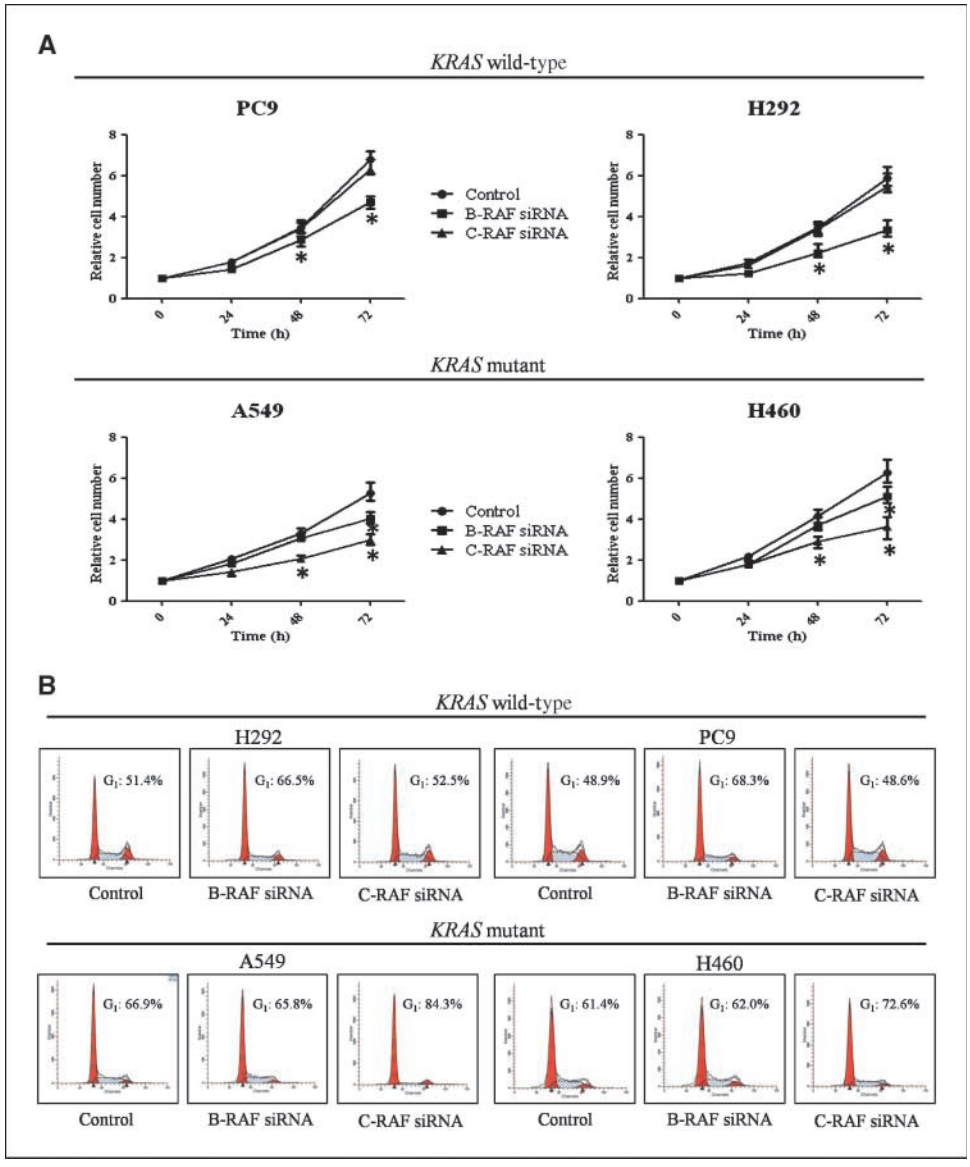

Figure 5. Effects of B-RAF or C-RAF depletion on cell proliferation and cell cycle distribution in NSCLC cells. *A,* cells harboring wild-type or mutant *KRAS* were transfected with nonspecific (control), B-RAF, or C-RAF siRNAs for the indicated times, after which the number of viable cells was determined by staining with trypan blue. The number of viable cells is expressed relative to the value for time 0. *Points,* mean values from three independent experiments; *bars,* SD. *, $P < 0.05$ versus the corresponding value for cells transfected with the nonspecific siRNA. *B,* cells were transfected as in *A* for 48 h, fixed, stained with propidium iodide, and analyzed for cell cycle distribution by flow cytometry. The percentage of cells in G_1 phase is indicated. Data are from representative experiments that were repeated on three separate occasions.

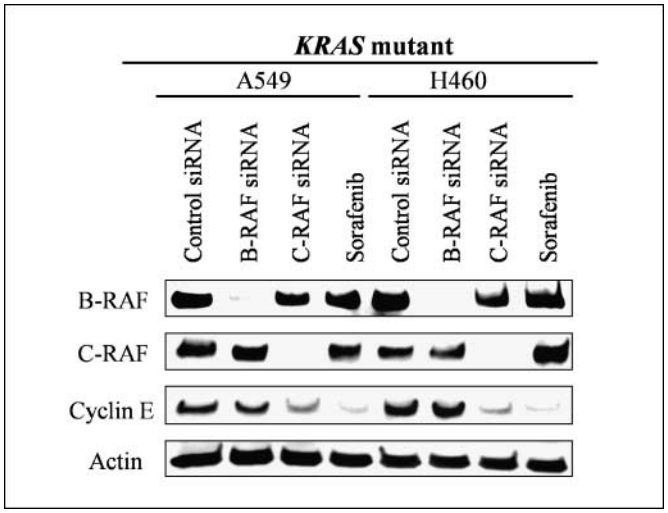

Figure 6. Effects of C-RAF depletion or sorafenib on cyclin E expression in NSCLC cells with mutant *KRAS*. Cells harboring mutant *KRAS* were transiently transfected for 48 h with nonspecific (control), B-RAF, or C-RAF siRNAs or were exposed to 15 μmol/L sorafenib for 24 h in complete medium. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to B-RAF, C-RAF, cyclin E, and β-actin.

proteins, our present data suggest that the observed down-regulation of cyclin E may contribute to the G_1 arrest induced by C-RAF depletion or by sorafenib in NSCLC cells with mutant *KRAS*.

Sorafenib inhibits several RTKs that participate in neovascularization, including vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-3 (16). Inhibition of angiogenesis might thus be expected to contribute to the inhibition of tumor growth by this drug in addition to its effects on RAF signaling. Although sorafenib was previously shown to inhibit the growth of a variety of human tumor xenografts in mice (13, 16, 46), it has been difficult to measure the relative contributions of its antiangiogenic activity and its direct antitumor activity mediated by RAF inhibition. In the present study, we have provided insight into the inhibitory effect of sorafenib on tumor cell growth *in vitro* that is mediated by inhibition of RAF signaling pathways. Our results suggest that sorafenib targets B-RAF in NSCLC cells with wild-type *KRAS* and C-RAF in those with mutant *KRAS*, and they provide a rationale for future clinical investigation of the therapeutic efficacy of sorafenib for NSCLC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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