Oncogenic BRAF Deletions That Function as Homodimers and Are Sensitive to Inhibition by RAF Dimer Inhibitor LY3009120

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INTRODUCTION

Somatic mutations in the BRAF gene were discovered in 2002 in melanoma, where they behave as potent oncogenes and activate downstream MAPK signaling and cancer cell growth (1–5). BRAF mutations have subsequently been found in many other tumor types, including thyroid, ovarian, colorectal, and non–small cell lung cancers, as well as in hairy cell leukemia and Langerhans cell histiocytosis (1, 6–9). In melanoma, the V600E hotspot mutation is particularly prevalent, but mutations affecting side chains other than valine 600 (non-V600 or atypical) have been described either adjacent to the activation segment (e.g., L597, G596, F595, and...
E586) or within the glycine-rich GXGXXG motif (e.g., G464, G466, and G469) of the kinase domain (1, 10). In patients with melanoma, atypical BRAF mutations were detected in 37 out of 499 (7.4%) patient specimens (11). In colorectal cancer samples with BRAF mutations, it has been reported that approximately 50% of them are V600E, and the remaining 50% are atypical mutations, i.e., D594V and T599I (12). BRAF mutations are found in 6.7% of lung adenocarcinomas, and 80% of these are atypical BRAF mutations (13). In addition to missense mutations, oncogenic BRAF fusions were also identified in cancers of the thyroid and prostate, melanoma, and astrocytomas (14–19). These fusions either encode protein partners that contribute coiled-coil (CC) or zinc-finger dimerization motifs to produce constitutively activated BRAF dimers, or remove at least the first eight exons of BRAF that are known to promote BRAF dimerization.

Recent mechanistic studies suggest that BRAFV600E protein functions as a monomer, whereas most of the atypical BRAF-mutant proteins function as dimers (5, 20–23). The ectopic expression of BRAFV600E induces constitutive activation of downstream ERK1/2 signaling, including negative feedback regulation of RTKs and RAS. Among BRAFV600E-expressing cells, BRAF monomer remains the primary driver of MEK1/2 and ERK1/2 signaling with minimal contribution from RTKs and RAS, thus making it an attractive anticancer target (4, 24). This led to the identification and FDA approval of the BRAF-selective inhibitors vemurafenib and dabrafenib. Both inhibitors showed antitumor activities in BRAF-mutant xenograft models (25–27), and significant clinical benefit among patients with BRAF-mutant melanoma (28–30). However, these first-generation BRAF drugs are not effective inhibitors of dimeric forms of RAF, including RAS-activated RAF dimers, many atypical BRAF mutants, BRAF splice forms, and BRAF fusions. Indeed, vemurafenib and dabrafenib have been shown to induce the dimerization of RAF proteins and promote paradoxical pathway activation of the MAPK pathway in BRAF wild-type (WT) cells (31–33). The paradoxical activation is thought to explain the promotion of tumor growth and metastasis observed with BRAF inhibitors in BRAF WT preclinical models (33, 34). Clinically, these compounds promote skin side effects including keratoacanthomas and squamous cell carcinomas (29, 30). We have recently developed LY3009120, a pan-RAF and RAF dimer inhibitor currently in clinical studies. LY3009120 is able to effectively inhibit active RAF dimers with minimal paradoxical activation (35, 36). In this study, we have discovered novel BRAF aberrant variants, which have in-frame deletions within or adjacent to residues L485-P490 of the αC-helix region in patient samples and cell lines of pancreatic, lung, and ovarian cancers. Further analyses revealed that the L485-P490-deleted BRAF is an activating
BRAF mutation, functions as a BRAF homodimer, and is able to transform the cells. Tumor cells with these BRAF deletions are resistant to the BRAF selective inhibitor vemurafenib but sensitive to the RAF dimer inhibitor LY3009120 in vitro and in vivo. LY3009120 represents a potential opportunity for treatment of patients with cancer with BRAF deletions or other atypical BRAF mutations where BRAF is activated as a dimer.

RESULTS

Identification and Confirmation of BRAF In-Frame Deletions within or Adjacent to Residues L485-P490 of the αC-Helix Region in Cancer Cell Lines and Patient Samples

In an unbiased screen of a large panel of tumor cell lines for their sensitivity to MAPK pathway inhibitors, we observed that BxPC-3 cells were very sensitive to the RAF dimer inhibitor LY3009120, but not sensitive to the BRAF-selective inhibitors vemurafenib or dabrafenib (36). BxPC-3 cells, derived from a pancreatic adenocarcinoma, are unusual for not having a KRAS mutation. As part of our Lilly internal genomics effort, we have genetically characterized the tumor cell line panel by whole exome sequencing (WES). When we carefully evaluated these data for mutations in RAS pathway genes in BxPC-3 cells, including small in-frame deletions which are easy to be ignored by routine data analysis, we discovered that BxPC-3 cells have a 5-amino acid deletion near the αC-helix region of the BRAF kinase domain (V487-P492Δ). One other cell line, NCI-H2405, also stood out in our tumor cell line profiling for being sensitive to LY3009120, but insensitive to BRAF-selective inhibitors, despite having no well-described mutations in RAS pathway genes (36). Close inspection of WES data revealed an in-frame deletion in H2405 affecting the same region of the BRAF gene in BxPC-3 cells (Table S1). Further searching of cell line databases identified one additional cell line, OV-90, an ovarian adenocarcinoma with a similar BRAF N486-P490 deletion, and sensitive to LY3009120, but not vemurafenib. These in-frame deletions were further verified and confirmed by Sanger sequencing (i.e., BxPC-3; Supplementary Fig. S1A–S1C).

These results hinted that the small in-frame deletions might explain the high sensitivity of these cells to LY3009120 and therefore would indicate a potential subset of patients who could benefit from this drug. To evaluate if these in-frame deletions occur in patients with cancer, we analyzed the publicly available databases from The Cancer Genome Atlas (TCGA) and The International Cancer Genome Consortium (ICGC). Indeed, several variations of these in-frame deletions were discovered in pancreatic cancer patient samples (T488-Q493ΔK, N486-Q490 deletion) and thyroid carcinoma samples (P490-Q494, T488-P492, N486-P490 deletion) from TCGA studies. Two pancreatic cancer patient samples harboring in-frame deletion variants (T488-Q493ΔK and N486-P490 deletion) were also found in ICGC studies (Supplementary Table S1). Further analysis revealed that these BRAF in-frame deletions are mutually exclusive from RAS and BRAFV600E mutations. In all above reported cases, no missense mutation in KRAS (G12, G13, Q61), NRAS (Q61), or BRAF (V600) was found. This is especially relevant in pancreatic cancers that have a high prevalence of KRAS mutation. Overall, the rate of BRAF deletion in patients with KRAS WT pancreatic cancer is 4.2% based on TCGA and ICGC studies (Fig. 1A). For thyroid carcinomas, 3 out of 506 patients (0.59%) were identified to have the BRAF in-frame deletion. These incidence rates are likely underestimated due to the technical challenge in detecting a deletion and the limitations of sequencing technologies.

BRAF In-Frame Deletions Activate MAPK Signaling in Tumor Cells and Ectopically Expressed Cells

To evaluate if these in-frame BRAF deletions activate downstream signaling, we conducted RAF isomorph-specific knockdown with siRNA in tumor cell lines H2405, BxPC-3, and OV-90. As demonstrated in Fig. 1B with H2405 cells, siRNA knockdown of BRAF alone, or combinations of BRAF and other RAF isomers, showed significant decreases in phospho-MEK and ERK. However, knockdown of ARAF or CRAF alone, or their combination, had minimal effects on phospho-MEK or ERK levels. Similar siRNA knockdown results were observed in BxPC-3 (Fig. 1C) and OV-90 (Fig. 1D) cells, although the degree of phospho-MEK and ERK inhibition was different among these cells, likely due to differences in BRAF knockdown efficiency. These results suggest that BRAF is a major isoform to activate MAPK signaling in these tumor cells. To further confirm pathway activation, we transfected a representative BRAF L485-P490 deletion (ΔBRAF) with or without the BRAF dimer–deficient mutation (BRAF R509H) into HEK293 cells. As shown in Fig. 1E, ΔBRAF caused significant elevation of phospho-MEK and ERK. Interestingly, the R509H mutation showed significantly reduced activation, suggesting that the BRAF deletion may function as a RAF dimer. In addition to activating MAPK signaling, BRAF in-frame deletions appear to be important for tumor cell proliferation. siRNA knockdown of BRAF alone showed significant inhibition of cell proliferation in H2405 (Fig. 1F), BxPC-3 (Fig. 1G), and OV-90 (Fig. 1H) cells. However, knockdown of either ARAF or CRAF had no statistically significant effect on cell proliferation as compared with controls. Overall, the MAPK activation and cell proliferation data suggest that the identified BRAF deletions are activating alterations and potentially oncogenic.

BRAF Deletions Transform Cells in a BRAF Dimer-Dependent Manner

To validate if BRAF deletions possess oncogenic transformation activities, mouse NIH/3T3 cells were stably transfected with ΔBRAF and then grown in soft agar culture. In three independent studies, ectopic expression of ΔBRAF was able to transform NIH/3T3 cells and promote colony formation in soft agar (Fig. 2A and Supplementary Fig. S2), whereas expression of WT BRAF revealed no transformation activity (Supplementary Fig. S2). As a positive control, the anchorage-independent growth of NIH/3T3 cells was also observed with the expression of BRAFV600E. Again, ΔBRAF with a R509H mutation did not support anchorage-independent growth in soft agar culture (Fig. 2A and B and Supplementary Fig. S2), indicating that ΔBRAF-promoted anchorage-independent growth is dependent on BRAF dimerization. Additionally, the ectopic expression of ΔBRAF in NIH/3T3 cells elevated MAPK signaling as evaluated by phospho-MEK and ERK levels, whereas a concomitant R509H mutation reduced phospho-MEK and ERK (Fig. 2C), consistent with the observation in HEK293 cells (Fig. 1E).
Figure 1. Prevalence of BRAF deletions in thyroid and pancreatic patient samples and BRAF dependency of MAPK activation in tumor cells and HEK293 cells harboring BRAF deletions. Overall, these results strongly suggest that ΔBRAF is an activating and oncogenic alteration, because the anchorage-independent growth is one of the hallmarks of cell transformation.

To further evaluate the role of the in-frame BRAF deletions in the transformation of tumor cells, H2405 and OV-90 were transfected with ARAF, BRAF, or CRAF siRNA and grown in soft-agar culture. On the one hand, as demonstrated in Fig. 2D–F, knockdown of BRAF resulted in a significant decrease in colony formation. On the other hand, knockdown of either ARAF or CRAF had minimal effect compared with the control. This suggests that BRAF plays the most important role among RAF isoforms in maintaining transformation activities in these tumor cells. It is also noteworthy that knockdown of KRAS showed minimal effects on transformation activities (Fig. 2G–I).
or MAPK signaling (Fig. 2J) in these BRAF deletion–expressing tumor cells, suggesting that these BRAF deletions function as activating mutations in a KRAS-independent manner.

**BRAF In-Frame Deletions Mainly Function as BRAF Homodimers**

To understand if BRAF deletions mainly function as BRAF homodimers or BRAF/CRAF heterodimers, we developed in situ proximity ligation assays (PLA) as described previously (37, 38). As a control for BRAF homodimers, we transfected A375 cells with a construct encoding the p61BRAF<sup>V600E</sup> splice variant. Consistent with previous observations that p61BRAF<sup>V600E</sup> mainly functions as a BRAF homodimer (5), the ectopic expression of p61BRAF<sup>V600E</sup> exhibits a strong in situ PLA signal for BRAF homodimers but minimal detectable BRAF/CRAF heterodimer signal (Fig. 3A). HeLa cells treated with EGF to induce BRAF/CRAF heterodimers served as a positive control for the ability of our PLA system to
detect this dimeric species (38). As revealed in Supplementary Fig. S3A, treatment of HeLa cells by EGF induced a clear PLA signal for BRAF/CRAF heterodimer. With PLAs for BRAF homodimers and BRAF/CRAF heterodimers established, we examined the status of RAF dimers in tumor cells. As shown in Fig. 3A, H2405, BxPC-3, and OV-90 cells harboring BRAF in-frame deletions all showed clear evidence for BRAF homodimers but not for BRAF/CRAF heterodimers, suggesting that the BRAF homodimer is the major RAF dimer in these cells (Fig. 3A and B).

Figure 3. BRAF in-frame deletions mainly function as BRAF homodimers. A, detection of BRAF/CRAF heterodimers and BRAF homodimers in H2405, BxPC-3, OV-90, and A375 cells ectopically expressing p61BRAF V600E using in situ PLA. B, quantification of in situ PLA signals of A (mean ± SEM). The number of PLA signals per cell with at least 1,000 cells for all reactions in triplicate was quantified and analyzed by Cellomics ArrayScan VTI Reader and HCS software (**, P < 0.01; ***, P < 0.001, one-tailed t test). C, detection of BRAF/CRAF heterodimers and BRAF homodimers in HEK293 cells ectopically expressing BRAF E586K, BRAF L485-P490 deletion (ΔBRAF) with or without R509H, or empty vector (control) using PLA. PLA signals (red spots) were examined under a confocal microscope and representative images were shown from at least two or three independent experiments. D, quantification of in situ PLA signals of C (mean ± SEM) in HEK293 expressing the indicated BRAF proteins. E, detection of BRAF homodimer by IP and Western blot analysis. HEK293 cells stably expressing FLAG-tagged ΔBRAF or ΔBRAF R509H protein were transfected with vectors encoding MYC-tagged ΔBRAF followed by IP using anti-FLAG or anti-MYC antibody-conjugated beads. The input and IP-prepared proteins were subjected to Western blot analysis with anti-FLAG and anti-MYC antibodies.

F, CRAF S338 phosphorylation and MAPK activation in ΔBRAF-transfected HEK293 and NIH/3T3 cells.
The existence of BRAF homodimers in tumor cells, and the demonstration that Arg509 is critical for signaling of these deletion mutants, indicated that the mutations may activate downstream signaling by promoting the formation of dimers just like many other oncogenic BRAF alterations. To test this idea, we transfected ΔBRAF into HEK293 cells to detect in situ dimerization using PLA. As a positive control, ectopic expression of BRAF<sup>E545K</sup> has been shown to promote RAF dimerization (22, 38, 39), exhibited a strong PLA signal for the BRAF homodimer (Fig. 3C). In contrast, BRAF homodimers were minimal in cells expressing CRAF<sup>E476K</sup>, a CRAF dimer–promoting mutation, serving as a negative control (Supplementary Fig. S3B). Similar to BRAF<sup>E545K</sup>, the ectopic expression of the BRAF predominately promoted BRAF homodimers, while a low level of BRAF/CRAF heterodimer signal was detected. The homodimer signal was substantially reduced by R509H BRAF dimer–BRAF/CRAF heterodimer signal was detected. The homodimer formation of BRAF homodimers in cells expressing BRAF<sup>E545K</sup>, and Hinder Its Flexibility by Locking the Helix in αC-helix-out conformations (Fig. 4A). Molecular modeling suggested that the in-frame deletions shorten the β3/αC-helix loop and hinder the flexibility of αC-helix by locking it in the αC-helix-in conformation via the Glu501/Lys483 salt bridge (Fig. 4B). Although this αC-helix-in conformation accommodates binding of type IIα inhibitors (e.g., LY3009120), it disfavors the type IIβ binders (e.g., vemurafenib) that require the αC-helix-out binding conformation. Meanwhile, the αC-helix-in conformation of BRAF promotes protein dimerization through the network of intermolecular interactions at the dimer surface, including multiple salt bridge/hydrogen bond interactions between Arg506 and Arg509, and Asp449 and Thr508, respectively (Fig. 4C). To summarize, the active αC-helix-in conformation stabilized by a shortened β3/αC-helix loop region in the BRAF in-frame deletion mutants favors BRAF dimerization, leading to MAPK pathway activation. This is consistent with previous reports suggesting that the αC-helix-in conformation of the RAF proteins promotes dimer formation (36, 40).

**BRAF Deletion-Mediated MAPK Activation Is Sensitive to LY3009120, a RAF Dimer Inhibitor, but Resistant to Vemurafenib**

Next, we investigated the sensitivity of the BRAF deletion-mediated MAPK activation to MAPK pathway inhibitors. As revealed in Fig. 5A, vemurafenib fails to reduce phospho-MEK or ERK, at concentrations below 10 μmol/L in all three cell lines—H2405, BxPC-3, and OV-90—that harbor BRAF deletions. Similarly, treatment with another BRAF-selective inhibitor, dabrafenib, revealed minimal effects on MEK and ERK phosphorylation in H2405 and BxPC-3 and modest inhibition in OV-90 (Supplementary Fig. S5A). In contrast, LY3009120 demonstrated potent and dose-dependent inhibition of phospho-MEK and ERK with significant inhibition observed at 0.01 μmol/L in all three cell lines (Fig. 5B). Similar to LY3009120, the MEK inhibitor trametinib is potent and active at inhibiting the phospho-MEK and ERK activities in these cells (Fig. 5C). These results further solidify the notion that the BRAF deletions function as RAF dimers. The sensitivities of BRAF-deleted cells to LY3009120 are similar to BRAF<sup>509E</sup> mutant A375 cells and are considerably more sensitive than KRAS-mutant HCT116 cells based on phospho-ERK inhibition (Fig. 5D and E) and cell proliferation (Supplementary Fig. S5B and S5C). To further verify these results, we compared the inhibitory activities of vemurafenib and LY3009120 in HEK293 cells transfected with ABRAF. Consistent with results obtained in H2405, BxPC-3, and OV-90 cells, LY3009120 exhibited dose-dependent inhibition of phospho-MEK and ERK in HEK293 cells (Fig. 5F). However, vemurafenib showed no inhibitory activity (Fig. 5G).

**Growth of Tumor Cells Harboring BRAF Deletion Is Sensitive to LY3009120, but Resistant to Vemurafenib In Vitro**

We then evaluated the in vitro growth inhibitory activities of MAPK pathway inhibitors to tumor cells harboring BRAF deletions. As shown in Fig. 6A–D, LY3009120 demonstrated a concentration-dependent cell growth inhibition with IC<sub>50</sub> values of 0.04, 0.087, and 0.007 μmol/L against H2405, BxPC-3, and OV-90 cells, respectively. However, vemurafenib had minimal activity inhibiting the cell growth of these cells. Again, the MEK inhibitor trametinib showed potent cell growth inhibition with
Figure 4. Molecular modeling of the BRAF in-frame deletion using protein coordinates from the BRAF/LY3009120 complex (PDB 5C9C). A, overall view of the BRAF in-frame deletion in complex with LY3009120. Key structural elements of the kinase domain and BRAF-deleted region are marked. The ATP-binding regions of ΔBRAF are shown in the following colors: G-loop, green; αC-helix, magenta; hinge, gold; DFG (aspartic acid, phenylalanine and glycine) motif, turquoise; catalytic loop, red. Pan-RAF type-IIa inhibitor LY3009120 is shown in a space-filled model and colored in the following atom colors: carbon, green; nitrogen, blue; oxygen, red; fluorine, light green. ΔBRAF is in the DFG-out/αC-helix-in conformation. All identified ΔBRAF in-frame deletions near L485-A489 (Supplementary Table S1) are located within the β3/αC-helix loop, which provides the essential flexibility to the αC-helix to toggle between the active (αC-helix-in) and inactive (αC-helix-out) conformations. B, superimposed view of the β3/αC-helix loop region in the ΔBRAF-mutant model (colored in gold) and WT BRAF (PDB 5C9C). Residue deletion segment L486-A489 is shown as sticks and colored in the following atom colors: carbon, green; nitrogen, blue; oxygen, red. Molecular modeling suggests that the in-frame deletions shorten the β3/αC-helix loop and impair its flexibility by locking the helix in the active αC-helix-in conformation via the Glu501/Lys483 salt bridge. C, surface representation of the dimeric ΔBRAF mutant viewed approximately down the local 2-fold axis. The αC-helix-in conformation of the mutant protein favors and promotes dimerization through the network of intermolecular interactions at the dimer interface, including multiple salt bridge/hydrogen bond interactions between R506 and R509 and D449 and T508, respectively.

IC50 values of 0.079, 0.006, and 0.003 μmol/L against H2405, BxPC-3, and OV-90 cells, respectively. Further cell-cycle analysis by flow cytometry illustrated that LY3009120 or trametinib treatment induced an increase of G1–G0 phase and a decrease of S phase in these cells (Fig. 6E–G). These cell-cycle effects were further verified by BrdUrd incorporation (Supplementary Fig. S6A). LY3009120 at 1 μmol/L or trametinib at 0.2 μmol/L significantly reduced BrdUrd-positive cells, whereas vemurafenib at 5 μmol/L had no effect in all three cell lines tested. In addition to cell-cycle G1–G0 arrest, LY3009120 or trametinib treatment also induced an increase of sub-G1 population of these cells, suggesting a compound-induced apoptotic effect (Fig. 6E–G). The apoptotic effects were further verified by LY3009120-induced increases of cleaved PARP in all three cell lines (Fig. 6H–J). This PARP cleavage can be inhibited by a pan-caspase inhibitor, Z-VAD-FMK, in a dose-dependent manner, suggesting that the
**Figure 5.** BRAF deletion–mediated MAPK activation is sensitive to LY3009120, a RAF dimer inhibitor, and trametinib, but resistant to vemurafenib, a BRAF monomer inhibitor. A–C, phospho-MEK and ERK levels of H2405, BxPC-3, and OV-90 cells treated with vemurafenib, LY3009120, or trametinib. Cells were treated at indicated concentrations for 2 hours, and cell lysates were analyzed for MEK and ERK phosphorylation by Western blotting. D and E, phospho-MEK and phospho-ERK inhibition of BRAFV600E-mutant A375 and KRASG12D-mutant HCT116 cells by LY3009120. F–G, phospho-MEK and phospho-ERK inhibition by LY3009120 and vemurafenib in ΔBRAF-transfected HEK293 cells. HEK293 cells stably expressing ΔBRAF were treated with LY3009120 or vemurafenib at indicated concentrations for 2 hours. Cell lysates were analyzed for MEK and ERK phosphorylation by Western blotting.
Oncogenic BRAF Deletions Functioning as Homodimers

**Figure 6.** Growth of tumor cells harboring BRAF deletion is sensitive to LY3009120, but resistant to vemurafenib in vitro. A–C, antiproliferation activities of vemurafenib, LY3009120, and trametinib in H2405, BxPC-3, or OV-90 cells assessed by the CellTiter-Glo assay. The cells were treated for 72 hours with different inhibitors at indicated concentrations. D, antiproliferation IC_{50} of vemurafenib (Vem), LY3009120 (LY), or trametinib (Tra) in H2405, BxPC-3, and OV-90 cells. IC_{50} was calculated via sigmoidal dose-response curve using GraphPad Prism 4 software. E–G, cell-cycle analysis by flow cytometry of H2405, BxPC-3, or OV-90 cells treated with vemurafenib (5 μmol/L), LY3009120 (1 μmol/L), or trametinib (0.2 μmol/L). Cells were subjected to PI staining at 72 hours after treatment. Dead cells are indicated as debris. Representative histograms are shown from three independent experiments. H–J, apoptosis analysis of H2405, BxPC-3, and OV-90 cells treated with vemurafenib (5 μmol/L) or LY3009120 (1 μmol/L) for 2, 24, and 48 hours, respectively. Cell lysates were analyzed for MEK and ERK phosphorylation and cleaved PARP (cPARP) induction by Western blotting.
apoptosis is caspase dependent (Supplementary Fig. S6B). In contrast to LY3009120, vemurafenib treatment had minimal effects on cell-cycle G1–G0 arrest or apoptosis of these tumor cells (Fig. 6E–J). Similar to vemurafenib, dabrafenib treatment did not induce apoptosis of H2405 or BxPC-3 cells based on cPARP (Supplementary Fig. S6C).

To extend the analysis of LY3009120 against tumor cells harboring atypical BRAF mutations where BRAF proteins mostly function as dimers, we tested 14 additional cell lines, including 2 PDX cell lines, BXF 1218L and RXL 1183L, as shown in Supplementary Table S2. LY3009120 is active against the majority of these tumor cell lines in vitro. Among them, 12 of 14 cell lines exhibited absolute IC50 values from 0.045 to 0.58 μmol/L LY3009120, whereas vemurafenib was generally inactive.

**Xenograft Tumors Harboring BRAF Deletions Are Sensitive to LY3009120, but Resistant to Vemurafenib In Vivo**

We then attempted to develop rat xenograft models with H2405, BxPC-3, and OV-90 cells. Both H2405 and BxPC-3 cells were able to grow tumors consistently, whereas OV-90 cells failed to grow tumors in nude rats. To assess the in vivo sensitivity to MAPK pathway inhibitors, we treated the xenograft tumors with LY3009120 or vemurafenib as described. As demonstrated in Fig. 7A and Supplementary Fig. S7A, in the H2405 xenograft model, treatment of LY3009120 at 15 or 30 mg/kg achieved almost complete tumor growth regression, whereas vemurafenib treatment at 20 mg/kg had no antitumor growth activity despite achieving significant single-agent activity in melanoma BRAF^{V600E}-mutant models (36, 41). Similarly, in the BxPC-3 xenograft model, LY3009120 at 15 or 30 mg/kg demonstrated significant tumor growth inhibition and partial regression, whereas vemurafenib had no antitumor effect (Fig. 7B, Supplementary Fig. S7B). Western blot analysis of the tumor lysates from these studies revealed that LY3009120 significantly inhibited phospho-MEK and phospho-ERK, whereas vemurafenib did not (Fig. 7C and D). Further analysis revealed that treatment of LY3009120 at 15 or 30 mg/kg inhibited downstream phospho-MEK and ERK by approximately 70% and 60%, respectively, in the H2405 model (Fig. 7E), and significant inhibition of phospho-MEK (61% at 15 mg/kg; 71% at 30 mg/kg) and phospho-ERK (66% at 15 mg/kg; 75% at 30 mg/kg) by LY3009120 was also observed in the BxPC-3 model (Fig. 7F). Based on the inhibition of tumor growth and downstream signaling, LY3009120 treatment at 15 mg/kg achieved nearly maximum effect. In both studies, LY3009120 appeared to be well tolerated at 15 and 30 mg/kg with no significant body weight loss (Supplementary Fig. S7C and S7D). Overall, the results from these in vivo studies are completely consistent with in vitro observations. Xenograft tumors with a BRAF deletion are sensitive to RAF dimer inhibitor LY3009120 and resistant to the BRAF monomer inhibitor vemurafenib. In both studies, the tumor growth inhibition induced by LY3009120 was correlated with phospho-MEK and ERK inhibition within the tumors.

**DISCUSSION**

BRAF inhibitors vemurafenib and dabrafenib are active in BRAF^{V600E}-mutant melanoma. However, these inhibitors are less active in cells expressing WT BRAF and paradoxically activate downstream RAF–MEK–ERK signaling and promote tumor growth in cells with activating RAS mutations (33, 34). Consequently, these inhibitors should be used with caution in patients whose tumors harbor a RAS mutation. Recent studies have revealed that these BRAF-selective inhibitors promote BRAF and CRAF dimerization, an essential step in the paradoxical pathway activation (31–33). It has now become more evident that vemurafenib and dabrafenib primarily bind one of the two protomers of the asymmetric RAF dimers and thereby fail to effectively inhibit downstream signaling (36). LY3009120 is a pan-RAF inhibitor that binds both protomers of RAF dimers and effectively inhibits downstream signaling (35, 36). Due to their distinct mechanisms of action, LY3009120, but not vemurafenib, is active against tumor cells with RAF in-frame deletions identified in this study and many other atypical BRAF mutations where BRAF functions as dimers (Table S2). Our data provide additional evidence that LY3009120 is a RAF dimer inhibitor.

In this study, we have discovered novel oncogenic BRAF in-frame deletions with a distinct activating mechanism dependent on BRAF dimer formation in human cancers. In addition to cell lines, the BRAF deletions were also identified in patients with pancreatic cancer or thyroid carcinoma with overall prevalence of 0.63% and 0.59%, respectively, and 4.2% frequency occurring in KRAS WT pancreatic cancer. The prevalence of BRAF deletions is likely underestimated because the current sequencing technologies and analytic tools are mainly designed for identification of point mutations and less favorable for identification of small in-frame deletions. In addition to BRAF in-frame deletions, other atypical BRAF mutations and BRAF fusions where BRAF functions as dimers also occur in many cancer types, including melanoma, lung, colorectal, and pancreatic cancers. In lung adenocarcinoma, the overall BRAF mutation frequency is approximately 6.7%, and 80% of these are atypical (13). In colorectal cancer, BRAF mutations are present in about 10% of patients, with 50% atypical (12, 13). Although the frequencies of these BRAF alterations in lung, colon, and other disease-related mortality of these cancers irrespective of their mutational subtype remains high: 158,000 and 50,000 deaths per year in the United States in lung cancer and colon cancer, respectively, which suggests a clear unmet medical need (42, 43). LY3009120 may have the potential for treatment of this unique patient population.

We found that BRAF deletions are mutually exclusive with RAS mutations, suggesting that BRAF deletions are potential oncogenes. Indeed, we have confirmed that they are activating and oncogenic alterations. In three cancer cell lines, BxPC-3, H2405, and OV-90, harboring BRAF deletions, siRNA knockdown of BRAF, but not CRAF or ARAF, significantly reduced phospho-MEK and ERK levels, and ectopic expression of ΔBRAF enhanced phospho-MEK and ERK activation in HEK293 and NIH/3T3 cells. Knockdown of BRAF by siRNA or inhibition by LY3009120 inhibited proliferation of tumor cells harboring BRAF deletions. Ectopic expression of ΔBRAF is able to transform NIH/3T3 cells and promotes anchorage-independent growth that is comparable with that caused by the BRAF^{V600E} mutation. Finally, xenograft models developed with tumor cells harboring a BRAF deletion are...
highly sensitive to inhibition by the RAF dimer inhibitor LY3009120, and the tumor growth inhibition is associated with downregulation of phospho-MEK and ERK. Overall, these data support the conclusion that the novel BRAF deletions are activating and oncogenic alterations.

We found that MAPK activation by BRAF deletions is dependent on homodimerization. Ectopic expression of ΔBRAF activated phospho-MEK and ERK in HEK293 and NIH/3T3 cells, and BRAF dimer-deficient mutation R509H significantly reduced MEK and ERK activation, suggesting that BRAF-engaged dimer is important for pathway activation. Similarly in soft-agar culture, BRAF deletion with a dimer-deficient R509H substitution failed to transform NIH/3T3 cells. In situ PLA demonstrated that the BRAF homodimer is the major RAF dimer formed in tumor cells or transfected HEK293 cells harboring these BRAF in-frame deletions, and co-IP analysis

Figure 7. Xenograft tumors harboring BRAF deletion are sensitive to LY3009120, but resistant to vemurafenib. A and B, antitumor activities of LY3009120 (LY) and vemurafenib (Vem) in H2405 (A) and BxPC-3 (B) models. Xenografts were treated with vemurafenib (20 mg/kg), LY3009120 (15 or 30 mg/kg), or vehicle twice daily for 3 to 4 weeks (8 animals per treatment group) and tumor volumes (mean ± SEM) were measured every 3 to 5 days. C and D, inhibition of phospho-MEK and phospho-ERK in tumor lysates. H2405 and BxPC-3 tumors were lysed following the completion of the treatment and analyzed with Western blotting for MEK and ERK phosphorylation. E and F, densitometric analysis (mean ± SEM, relative to vehicle groups) of the levels of phospho-MEK and phospho-ERK in H2405 and BxPC-3 tumors after normalized to total MEK and ERK using ImageJ software (****, P < 0.0001, one-tailed t test).
revealed that the BRAF deletion is able to form BRAF homodimers. Structural analysis revealed that these BRAF deletions shorten the β3/αC-helix loop and hinder its flexibility by locking the helix in the active αC-helix-in conformation that favors BRAF dimerization. Finally, tumor cells with these BRAF deletions are sensitive to the RAF dimer inhibitor LY3090120 but resistant to the BRAF monomer inhibitor vemurafenib in vitro and in vivo. As indirect evidence, transfection of ABRF into HEK293 or NIH/3T3 cells activated the MAPK activity in a CRAF-independent manner. All together, these data suggest that these BRAF deletions function as BRAF homodimers.

In the original description of BRAF mutations in cancer, BRAFV600E was only 1 of the 14 BRAF alterations identified in cell lines and primary tumor samples (1). Since then, nearly 300 distinct missense mutations have been found in tumor samples and cancer cell lines (44). These missense mutations encompass over 100 of the 766 BRAF amino acids, but most of the mutations occur in the activation loop (A-loop) near V600, or in the phosphate-binding loop (P-loop) at residues 464–469 (10). According to a model based on crystal structures of the BRAF kinase domain, both loops interact with each other via hydrophobic interactions (2). Disruption of this interaction by V600 mutations results in a conformational change within the kinase domain and full activation of BRAF. Many non-V600 oncogenic variants of BRAF have been shown to activate signaling by promoting the formation of active dimers, and our data suggest that BRAF deletions also promote dimer formation. Structural analysis suggests an explanation for this ability and points to a novel mechanism of dimer promotion. The BRAF deletions described here all have a 5-amino acid deletion just outside the P-loop, within the β3/αC-helix loop, a region at the interface critical for RAF dimerization. The β3/αC-helix loop provides flexibility to the αC-helix, allowing movement between the active (αC-helix-in) and inactive (αC-helix-out) conformation. The 5-amino acid deletion shortens the β3/αC-helix loop, impairs its flexibility and fixes the helix in the active αC-helix-in conformation. The αC-helix-in conformation of the BRAF proteins favors and promotes BRAF dimerization as described (36, 40). Consistent with this model, tumor cells with BRAF deletions are sensitive to the RAF dimer inhibitor LY3090120, but resistant to the BRAF monomer inhibitor vemurafenib in vitro and in vivo. In principle, the structural studies do not preclude the possible stabilization of BRAF heterodimers with BRAF or CRAF. However, we have not found evidence for a substantial contribution of heterodimers. For example, transfection of ABRF into HEK293 or NIH/3T3 cells activated the MAPK activity in a CRAF-independent manner. The activation mechanism proposed here is consistent with recent findings showing that N-terminally truncated BRAF proteins, and many other atypical BRAF mutations, function as BRAF homodimers (5, 23).

The activating effect of the BRAF deletion described here highlights a region of the kinase domain that could play an important role in the normal control of RAF activity. In addition to BRAF, similar 5-amino acid deletions near the αC-helix domain of a protein kinase were also identified in other targets, such as EGFR and HER2. In EGFR, the exon 19 deletions including E746-A750 were characterized in non–small cell lung cancer (45, 46). For HER2, another EGFR family member, the L755-T799 deletion was recently identified in breast cancer (47). All these deletions have been found to be activating mutations. Therefore, the activating mechanism of BRAF deletions identified in this study may represent a common mechanism for activating other protein kinases.

**METHODS**

**Cell Culture, Antibodies, and Reagents**

BxPC-3, H2405, OV-90, A375, HCT116, NIH/3T3, and HEK-293 cells were obtained from the ATCC from 2010 to 2013 and stored within a central cell bank that performs cell line characterizations. All these cell lines were passaged for less than 2 months, after which new cultures were initiated from vials of frozen cells. Characterization of the cell lines was done by a third-party vendor (RADIL), which included profiling by PCR for contamination by various microorganisms of bacterial and viral origin. As a result, no contamination was detected. The samples were also verified to be of human origin without mammalian interspecies contamination. The alleles for 9 different genetic markers were used to determine that the banked cells matched the genetic profile that has been previously reported. H2405 cells were grown in ACL-4 medium (ATCC), whereas NIH/3T3, HEK-293, and A375 cells were maintained in DMEM supplemented with 10% FBS (Invitrogen). HCT116 cells were cultured in McCoy’s 5A with 10% FBS (Invitrogen), and BxPC-3 cells were cultured in RPMI with 10% FBS. OV-90 cells were grown in a 1:1 mixture of MCDB 105 medium containing a final concentration of 1.5 g/L sodium bicarbonate and Medium 199 containing a final concentration of 2.2 g/L sodium bicarbonate with 15% FBS (Thermo Scientific). The BRAF-selective inhibitor vemurafenib, the pan-RAF inhibitor LY3090120, and the MEK inhibitor trametinib were synthesized by Eli Lilly and Company. All siRNAs were obtained from Dharmacon (ON-TARGETplus siRNA). siRNA transfections were performed using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer’s instructions. All plasmids were created using standard cloning methods with pcDNA3.1 (Invitrogen) as a vector. All plasmid transfections were carried out using FuGENEHED transfection reagents (Promega) as per the manufacturer’s instructions.

**Deletion Detection from Sequencing Analysis**

BRAF deletion calls on cancer cell lines were aggregated by searching through the repositories COSMIC v71 and Sanger Institute’s Cancer Cell Line Project, and exome sequencing variant calls from Broad Institute’s Cancer Cell Line Encyclopedia (CCLE), as well as internally generated exome sequencing data. Internal exome data were prepared using Agilent Sure-Select 38 Mb all-exon capture library sequenced on the Illumina HiSeq 2000 platform, generating approximately 80x paired-end reads. Variant calling on internal exome and CCLE exome data were performed using the BWA-mem aligner v0.7.4 (mapped to GRCh37) and called with GATK lite v2.3, Freebayes v0.9.10, and Samtools mpileup v0.1.19. TCGA mutation data (MAF files) were downloaded from the Broad Institute’s GDAC firehose (2014_10_17 release). RNA-sequencing data (fastq files) from TCGA were downloaded from http://cghub.ucsc.edu under controlled access in accordance with the data-user agreement, and mapped to human genome GRCh37 using the GSNAP (2013-11-27) aligner. BRAF deletions based on RNA-sequencing data from TCGA and CCLE were identified by searching for reads with at least 2 base deletions in the BRAF genomic region, and analysis was done to determine the consequence of the change if it resulted in an in-frame deletion. For TCGA pancreatic and thyroid cancer samples, we further confirmed the deletion identified by searching through the mapped reads from the whole exome sequencing data where there were cases in which the deletion was not reported from the mutation data downloaded from Broad’s firehose. The ICGC data (release 17) was accessible from http://dcc.icgc.org.

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Preparation of Cell Lysates, Western Blot Analysis, and Cell Proliferation Assay

Cell lysate preparation, Western blot analysis, and cell proliferation assay were performed as described previously (41, 48).

Transfections and Immunoprecipitation

A375 cells transfected with BRAF<sup>V600E</sup> with deleted amino acids 169-380 (A375 p61V600E) and HEK293 transfected with BRAF<sup>E586K</sup>, CRAF<sup>478K</sup>, ΔBRAF, and ΔBRAF R509H were generated using pcDNA3.1 vectors under G418-containing medium selection and evaluated for BRAF and FLAG or MYC-tagged protein expression as described previously (36). For coimmunoprecipitation, HEK293 cells stably expressing FLAG-tagged ΔBRAF or ΔBRAF R509H proteins were transfected with pcDNA3.1 vector encoding MYC-tagged ΔBRAF followed by immunoprecipitation using anti-FLAG (Sigma) or anti-MYC magnetic beads (Cell Signaling Technologies). The IP-prepared proteins were next subjected to Western blot analysis as previously described (36).

Colony Transformation Assay in Soft-Agar Culture

For ectopic expression, NIH/3T3 cells transfected with ΔBRAF, ΔBRAF with R509H, BRAF<sup>V600E</sup>, or BRAF WT constructs or parental vector (pcDNA3.1) were selected in G418-containing medium for 2 weeks. The transfected cells (3 × 10<sup>4</sup>) in growth media with 0.3% agar were plated on top of 0.5% agar medium in 6-well tissue culture plates. Formation of spherical colonies was evaluated after 3 weeks under a microscope. For target protein knockdown with siRNAs, the transformation assay for H2405 and OV-90 cells was performed with the same procedure at 48 hours after transfection.

In Situ Proximity Ligation Assay

PLA was conducted and validated according to the manufacturer’s instructions (Olink Bioscience) as previously described (37, 38). Briefly, cells grown on glass slides or 96-well plates were fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100 before being incubated with 1% BSA-blocking solution overnight at 4°C. For detection of BRAF homodimers, monoclonal BRAF antibodies were first conjugated to PLUS and MINUS PLA oligonucleotides using the Duolink II Probes maker system (37). For detection of BRAF and CRAF heterodimers, the primary antibodies were directly used and followed by incubation with PLUS and MINUS oligonucleotide-conjugated PLA probes (38). The bound proximity probes were then visualized as red spots with Duolink In Situ Detection Reagents Orange (Olink Bioscience) and detected under a confocal fluorescence microscope. The nuclear staining with Hoechst 33342 was analyzed in each 96-well plate for all reactions in triplicate.

Cell-Cycle Analysis

Cell-cycle analysis was performed as described previously (36, 41). Cells treated with DMSO or inhibitors for 72 hours were harvested and fixed in 70% ethanol for 30 minutes at -20°C. After being washed with PBS, fixed cells were stained with propidium iodide/Triton X-100 staining solution and incubated for 30 minutes at room temperature. Fixed cells were then subjected to flow cytometric analysis on a Beckman Coulter FC 500 Cytomics flow cytometer. Data were analyzed with ModFit LT 3.0 (Verity House Software).

BrdUrd Incorporation Assay

Tumor cell lines H2405, BxPC-3, and OV-90 were grown in 6-well plates, and the growth medium was changed and the cells were treated with DMSO or inhibitors the following day. One hour prior to the end of the 72 hours of treatment time, cell medium was spiked with 10 μmol/L BrdUrd. After 1 hour of incubation with BrdUrd, cells were harvested and fixed in 70% EtOH at -20°C. Cells were then washed with PBS/BSA, incubated with 2N HCl/FBS for 20 minutes, and treated with sodium borate. To determine the amount of BrdUrd incorporation, cells were stained with isotype control or FITC-conjugated anti-BrdUrd antibody (BD Pharmingen) for 30 minutes, washed with PBS/BSA, and incubated in propidium iodide (Life Technologies) for 30 minutes before reading on a flow cytometer. Data were analyzed with FlowJo software.

In Vivo Xenograft Studies

In vivo studies were performed in accordance with the American Association for Laboratory Animal Care institutional guidelines. All the experimental protocols were approved by The Eli Lilly and Company Animal Care and Use Committee. Briefly, 5 × 10<sup>6</sup> to 10 × 10<sup>6</sup> tumor cells in a 1:1 Matrigel mix (0.2 mL total volume) were injected subcutaneously into the right hind flank of female NIH nude rats (Taconic Biosciences). After tumors reached a desired size of approximately 300 mm<sup>3</sup>, animals were randomized into groups of 8 for efficacy studies. Drugs (1X3009120 or vemurafenib) were administered orally (gavage) in 0.6-mL volume of vehicle with the dose schedules described in each study. Tumor growth and body weight were monitored over time to evaluate efficacy and signs of toxicity as described (41).

Disclosure of Potential Conflicts of Interest

Y.G. Yue is Director, Computational Biology, at Boehringer Ingelheim. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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Oncogenic BRAF Deletions Functioning as Homodimers


Oncogenic *BRAF* Deletions That Function as Homodimers and Are Sensitive to Inhibition by RAF Dimer Inhibitor LY3009120

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