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BRAF Mutants Evade ERK-Dependent Feedback by Different Mechanisms that Determine Their Sensitivity to Pharmacologic Inhibition

Highlights

- All activating BRAF mutants are RAS independent
- Activating BRAF mutants signal as active monomers or constitutive dimers
- RAF dimers are insensitive to clinical RAF inhibitors due to negative cooperativity
- A compound that equally inhibits mutant BRAF monomers and dimers overcomes resistance

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In Brief

Yao et al. show that RAS-independence is a common property of activating BRAF mutants that occurs by either of two mechanisms. Active monomers are sensitive to current drugs, whereas constitutive dimers are not. A compound that equally binds both sites of dimers is active against all activated BRAF mutants and overcomes acquired resistance to current drugs.

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BRAF Mutants Evade ERK-Dependent Feedback by Different Mechanisms that Determine Their Sensitivity to Pharmacologic Inhibition

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SUMMARY

ERK signaling requires RAS-induced RAF dimerization and is limited by feedback. Activated BRAF mutants evade feedback inhibition of RAS by either of two mechanisms. BRAF V600 mutants are activated monomers when RAS activity is low; all other activating BRAF mutants function as constitutive RAS-independent dimers. RAF inhibitors effectively inhibit mutant monomers, but not dimers; their binding to one site in the dimer significantly reduces their affinity for the second. Tumors with non-V600E BRAF mutants are insensitive to these drugs, and increased expression of BRAF V600E dimers causes acquired resistance. A compound that equally inhibits both sites of mutant RAF dimers inhibits tumors driven by either class of mutants or those BRAF V600E tumors with dimer-dependent acquired resistance to monomer-specific inhibitors.

INTRODUCTION

The oncogenic activation of ERK signaling output is characteristic of many cancers. Physiologic activation of the pathway occurs when upstream signals stimulate the binding of RAS to guanosine triphosphate (GTP) (Luo et al., 1996; Rajakulendran et al., 2009). Activated RAS binds to RAF family members and causes their homo- and heterodimerization and activation (Freeman et al., 2013). This in turn initiates the MEK/ERK kinase cascade and phosphorylation of effectors of the pathway by ERK. Activation of ERK also causes an array of negative regulatory events that serve to inhibit the pathway. ERK phosphorylates and inhibits receptors (Avraham and Yarden, 2011), the RAS guanosine diphosphate-GTP exchange factor SOS (Dong et al., 1996), and wild-type (WT) CRAF and BRAF (Dougherty et al., 2005). It also increases the expression of members of the Sprouty and DUSP families of proteins that inhibit the pathway (Pratilas et al., 2009). The former inhibit RTK activation of RAS, whereas the latter are MAPK phosphatases (Lang et al., 2006). Thus, negative feedback limits the amplitude and duration of the ERK signal.

Oncogenic mutations of NRAS, KRAS, BRAF, MEK1, and NF1 drive the ERK-dependent growth of many human cancers. These mutations activate both downstream signaling and potent negative feedback, as evidenced by reactivation of upstream and parallel components of the pathway in cells exposed to MEK or RAF inhibitors (Corcoran et al., 2012; Lito et al., 2012; Montero-Conde et al., 2013). We hypothesize that the elevated signaling output necessary for transformation requires selection of oncoproteins

Significance

We show that a fundamental property of activating BRAF mutants is their RAS independence. The mechanism whereby this occurs determines their sensitivity to current RAF inhibitors. These drugs potently inhibit signaling driven by active RAF monomers (BRAF V600) but not dimers. These findings can be used to characterize BRAF mutants detected in tumors and guide their treatment. A compound that is unaffected by induction of negative cooperativity inhibits signaling driven by activated mutant BRAF dimers or monomers and at doses lower than those required to inhibit wild-type RAF signaling. Such drugs may be useful in treating any tumor driven by an activating BRAF mutation and could supplant current "BRAF-monomer" inhibitors, which are limited by dimer-driven acquired resistance.

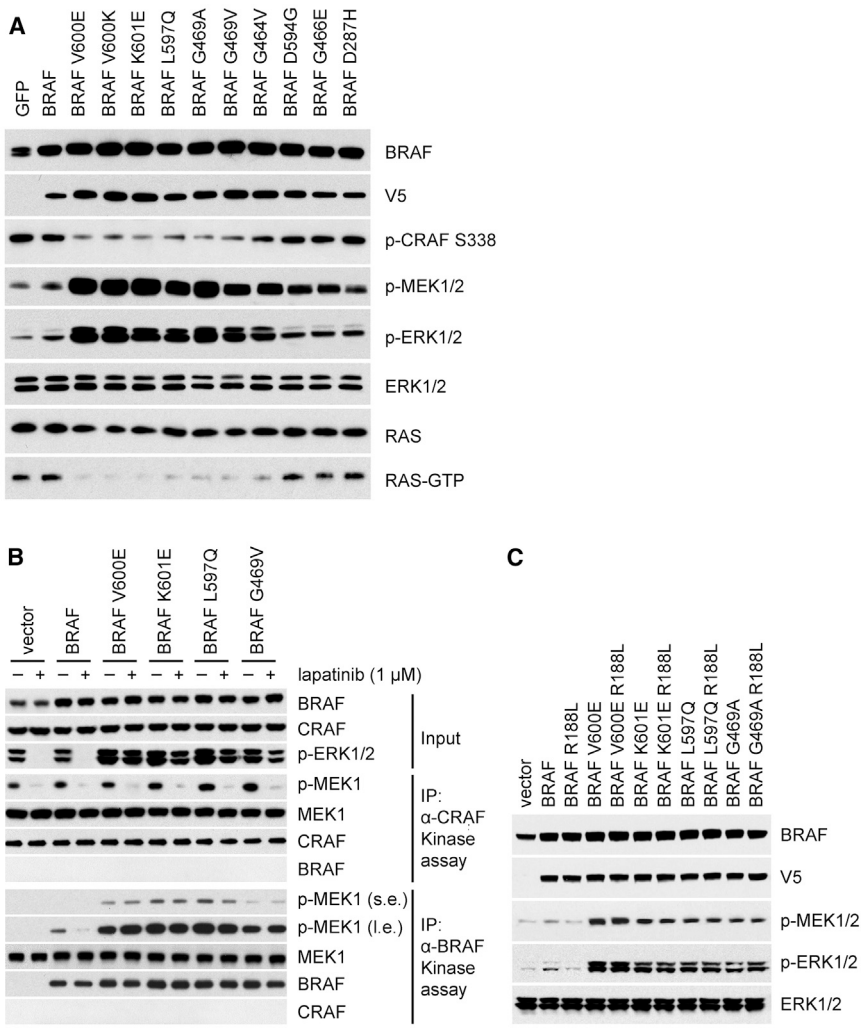


Figure 1. Activating BRAF Mutants Signal in a RAS-Independent Manner

(A) NIH 3T3 cells stably transduced with retrovirus carrying doxycycline-inducible WT BRAF or the indicated mutants were treated with doxycycline (30 ng/ml) for 24 hr. Expression and/or phosphorylation of the indicated proteins was assayed by western blot. Cellular RAS-GTP levels were determined using the active RAS pull-down assay.

(B) SKBR3 cells transiently expressing V5-tagged WT BRAF or the indicated mutants were treated with lapatinib (1 μM) or DMSO for 1 hr. Cell lysate from each sample was divided into two portions for immunoprecipitation with either anti-V5 antibody or anti-CRAF antibody, followed by an in vitro kinase assay with 0.5 μg K97R MEK1 protein. Five percent of the whole-cell extracts were used for immunoblot (input panels). l.e., longer exposure, s.e., short exposure.

(C) Ectopic expression of V5-tagged WT BRAF or the indicated mutants were expressed in SKBR3 cells followed by lapatinib treatment (1 μM for 1 hr). ERK signaling was assessed by western blot, as in (A).

See also Figure S1.

with decreased sensitivity to negative feedback. Mutations and translocations of RAF family genes are common in human tumors (Wan et al., 2004; Davies et al., 2002; Palanisamy et al., 2010). V600E is the most common *BRAF* mutation, but non-V600E mutations account for more than 50% of the *RAF* mutations in lung cancers (Paik et al., 2011) and occur in many other tumors. The kinase activity of many of these mutants has been shown to be activated compared with WT, but some BRAF mutations are kinase dead or have lower activity than WT BRAF (Wan et al., 2004). RAF fusions and truncations in which part of the amino-terminal domain of RAF is usually deleted also occur rarely in many tumors and in a high percentage of pilocytic astrocytomas (Berghoff and Preusser, 2014). Here, we ask how activating mutations of RAF hyperactivate signaling in the setting of ERK-dependent feedback inhibition of RAS.

RESULTS

Activating Mutants of BRAF Hyperactivate ERK Signaling and Suppress RAS Activation

RAS-GTP is suppressed in BRAF V600E tumor cells by ERK-dependent feedback (Lito et al., 2012). We asked whether this is a general property of tumor cells with activated mutant BRAFs. Levels of RAS-GTP and CRAF phosphorylation at serine 338 (pCRAF S338, a marker of CRAF activation) (Mason et al., 1999) were much lower in tumor cells with activating BRAF V600E, K601N, L597V, L597R, or G469A mutations than in those with WT RAF (Figure S1A). In contrast, levels of phosphorylated MEK (p-MEK) are highly elevated in RAF mutant cells even compared to those with mutant RAS. These results suggest that MEK is activated but RAS and CRAF are feedback inhibited in tumor cells with activating BRAF mutants.

To determine whether suppression of RAS activity is a general property of these mutants, we expressed mutant BRAFs in an inducible fashion in NIH 3T3 cells (Figure 1A). Induction of the expression of BRAF V600E, V600K, K601E, L597Q, G469A, G469V, or G464V to levels comparable to those of endogenous BRAF caused significant induction of p-MEK and p-ERK and marked inhibition of RAS-GTP and pCRAF S338. Induction of WT BRAF caused minor increases in p-MEK and p-ERK and had no effect on RAS-GTP or pCRAF S338. Thus, activated BRAF mutants significantly increase ERK signaling despite causing feedback inhibition of RAS activity to almost undetectable levels. By contrast, kinase-dead BRAF D594G and low-activity BRAF G466E and D287H marginally activated ERK signaling and didn't inhibit RAS function. These mutants may function in a fundamentally different way than the activating mutants, as previously suggested (Heidorn et al., 2010). In this paper, we focus on activating mutants of BRAF and how they function in cells in which RAS is feedback inhibited.

Table 1. Properties of WT and Mutant BRAF Alleles

BRAF Alleles	Feedback Inhibition of RAS-GTP	RAS Dependency of Kinase Activation	Sensitivity of R509H-Mediated Inhibition	Sensitivity to Vemurafenib	RAS Dependency of Dimerization with CRAF	RAS Dependency of Homodimer Formation
WT	N	Y	Y	N	Y	Y
V600E	Y	N	N	Y	Y	Y
V600K	Y	N	N	Y	Y	Y
V600D	Y	N	N	Y	Y	Y
V600R	Y	N	N	Y	Y	Y
V600M	Y	N	N	Y	Y	Y
K601E	Y	N	Y	N	Y	N
K601N	Y	N	Y	N	Y	N
K601T	Y	N	Y	N	Y	N
L597Q	Y	N	Y	N	Y	N
L597V	Y	N	Y	N	Y	N
G469A	Y	N	Y	N	Y	N
G469V	Y	N	Y	N	Y	N
G469R	Y	N	Y	N	Y	N
G464V	Y	N	Y	N	Y	N
G464E	Y	N	Y	N	Y	N
KIAA1549-BRAF	Y	N	Y	N	Y	N
p61 WT	Y	N	Y	N	Y	N
p61 V600E	Y	N	N	N	Y	N

Activation of Signaling by Hyperactivated RAF Mutants Is RAS Independent

Our findings imply that activated BRAF mutants signal in a RAS-independent manner and are thus insensitive to upstream feedback. To test this hypothesis, we used the *ERBB2* amplified breast cancer cell SKBR3 in which RAS activity and ERK signaling are potently suppressed after 1 hr exposure to the HER2 inhibitor lapatinib (Figure S1B). This system allows us to assess the RAS dependence of exogenously expressed RAF mutants. Lapatinib inhibited ERK signaling in SKBR3 cells overexpressing WT BRAF but failed to do so in those that express activating BRAF mutants (Figures 1B and S1C). Moreover, when CRAF or BRAF were immunoprecipitated from these cells, BRAF-associated kinase activity was sensitive to lapatinib only in cells expressing WT BRAF, not in those expressing mutant BRAF (Figures 1B and S1C). In contrast, CRAF-associated kinase activity was sensitive to lapatinib in all cells. Thus, the kinase activity of activating BRAF mutants is RAS independent. To exclude the possibility that their activity requires low levels of residual RAS-GTP, the R188L mutation that disrupts the RAS-BRAF interaction (Fabian et al., 1994) was introduced into the activating BRAF mutants and did not affect their ability to drive signaling (Figure 1C). To further confirm that these activating BRAF mutants signal in a RAS-independent manner, we used conditional RAS-less mouse embryo fibroblasts (MEFs) (Drosten et al., 2014). Knockout (KO) of *Kras*, the only RAS gene in these cells, causes their proliferative arrest and abrogates ERK signaling, but they remain viable. All of the activating BRAF mutants rescue MEK/ERK phosphorylation in these cells, but WT BRAF does not (Figure S1D). We have tested 16 activating BRAF mutants (Table 1), and all signal in a RAS-independent manner.

RAS-Independent BRAF Mutants Fall into Two Classes: Active Constitutive Dimers and Mutants that Are Active as Monomers in Cells with Low RAS Activity

Previous data suggested that V600E BRAF can signal as a monomer and is thus RAS independent (Poulikakos et al., 2011). We asked whether this is the general mechanism that underlies the RAS independence of activating RAF mutants. To address this question, we used the R509H and R401H/A mutations that impair dimerization of BRAF and CRAF, respectively, and eliminate their kinase activity (Poulikakos et al., 2010). R509H or R401H were introduced into BRAF or CRAF and multiple BRAF and CRAF mutants or truncations and expressed in SKBR3 cells (Figure 2A). ERK signaling in SKBR3 cells expressing these mutants was assessed after lapatinib treatment (low RAS-GTP state). ERK signaling was undetectable in cells when WT BRAF was overexpressed. Low levels of p-ERK were detected in cells overexpressing CRAF, but not in cells with CRAF 401H. The effects of R509H on ERK signaling were tested in 15 different activated BRAF mutants that were identified in human tumors (Figure 2A; Table 1). ERK signaling driven by 11 of the 15 was reduced to very low levels. The 4 mutants unaffected by the R509H were all V600 mutants (V600E/K/D/R). In contrast, the activities of K601 mutants, including K601E, are abrogated by the R509H mutation.

The results suggest that in cells with low RAS activity, BRAF V600 mutants signal as active monomers, whereas all other BRAF-activating mutants signal as constitutive, RAS-independent dimers. We confirmed this conclusion in the RAS-deficient MEFs. BRAF V600 R509H mutants restore ERK signaling in these cells, but R509H mutants of non-V600 BRAF mutants do not (Figure S2A).

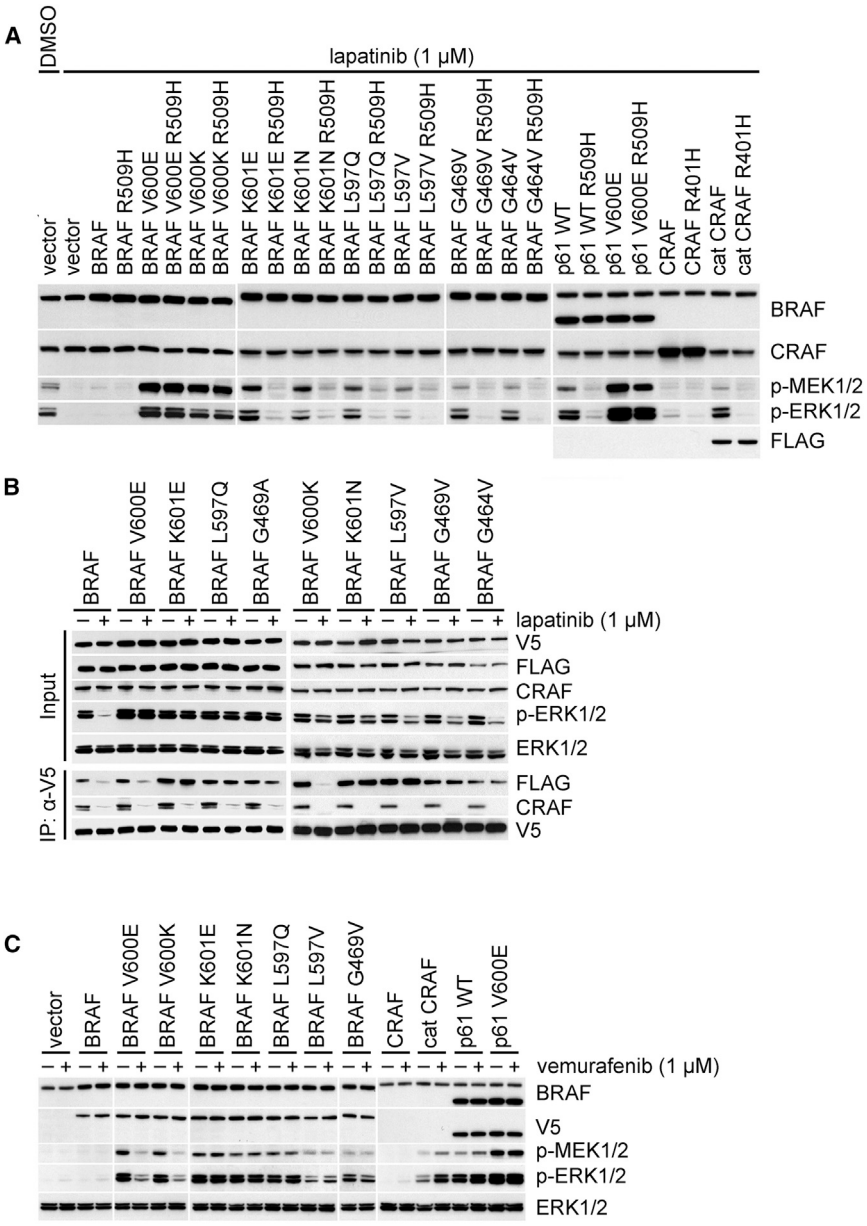


Figure 2. Activated RAF Proteins that Signal as Dimers Are Resistant to Vemurafenib

(A) SKBR3 cells were transfected with the indicated plasmids. After 24 hr, the cells were treated with 1 μM lapatinib for 1 hr. Cell lysates were then analyzed by western blot.

(B) SKBR3 cells transiently co-expressing Flag-tagged and V5-tagged WT or mutant BRAF proteins were treated with lapatinib (1 μM for 1 hr). BRAF dimerization was determined by immunoprecipitation.

(C) SKBR3 cells were transfected with the indicated plasmids. After 24 hr, the cells were treated with 1 μM lapatinib for 1 hr, followed by 1 μM vemurafenib for 1 hr. The indicated endogenous or ectopic proteins were assayed by western blot.

See also Figure S2.

We asked whether the non-V600 BRAF-activating mutants function as BRAF homodimers or BRAF/CRAF heterodimers. When these mutants were expressed in *Raf1*, which encodes CRAF, KO MEFs, they all potently activate ERK signaling and remain sensitive to R509H (Figure S2B). Thus, CRAF is not required for activation of ERK signaling by BRAF mutants that signal as constitutively active dimers. We evaluated the ability of these BRAF mutants to homodimerize and to heterodimerize with CRAF as a function of cellular RAS activity. We co-expressed V5- and FLAG-tagged BRAF mutants in SKBR3 cells and assessed co-precipitation of V5 with either FLAG (mutant BRAF homodimers) or CRAF (mutant BRAF/WT CRAF heterodimers) (Figure 2B). In control cells with adequate RAS-GTP levels, either WT BRAF or BRAF mutants form both BRAF homodimers and BRAF/CRAF heterodimers. One hour after inhibition of RAS activity with lapatinib, BRAF/CRAF heterodimers were lost from all cells. BRAF homodimers were lost in cells overexpressing WT BRAF or BRAF V600 mutant alleles, but ERK phosphorylation was abrogated only in the former (Figure 2B). Thus, the formation of BRAF V600E/K homodimers is RAS dependent, but both BRAF V600E/K dimers and monomers can activate ERK signaling. In contrast, dimerization and activity of non-V600 BRAF mutants are RAS independent (Figure 2B; Table 1). We conclude that activating BRAF mutants evade feedback by either of two mechanisms: the dimerization of V600 mutants remains RAS dependent, but their activity is not dependent on dimerization. Monomers of all non-V600 BRAF-activating mutants are inactive, and they all signal as active RAS-independent homodimers.

In cancer, RAF kinase is also activated by a variety of translocations and aberrant splice forms that encode fusion or truncated proteins in which an N-terminal domain containing the RAS-binding site is almost always deleted (Figure S2C). Engineered deletion of this domain has been shown to result in constitutive dimerization and activation (Cutler et al., 1998), so it seems likely that these fusions are also activated in this way. Cat C, an engineered N-terminal deletion of CRAF, and p61 WT BRAF, an engineered N-terminal deletion of BRAF, are activated constitutive dimers whose activity is abrogated by the R401H and R509H mutants respectively (Figure 2A). Similarly, ESRP1-CRAF and KIAA1549-BRAF are tumor-derived fusion proteins in which the N-terminal domain of CRAF or BRAF has been replaced by the fusion partner (Figure S2C). They also activate ERK signaling in a RAS-independent manner, and their activity is abrogated by the R401A and R509H mutations, respectively (Figure S2D). Thus, fusions

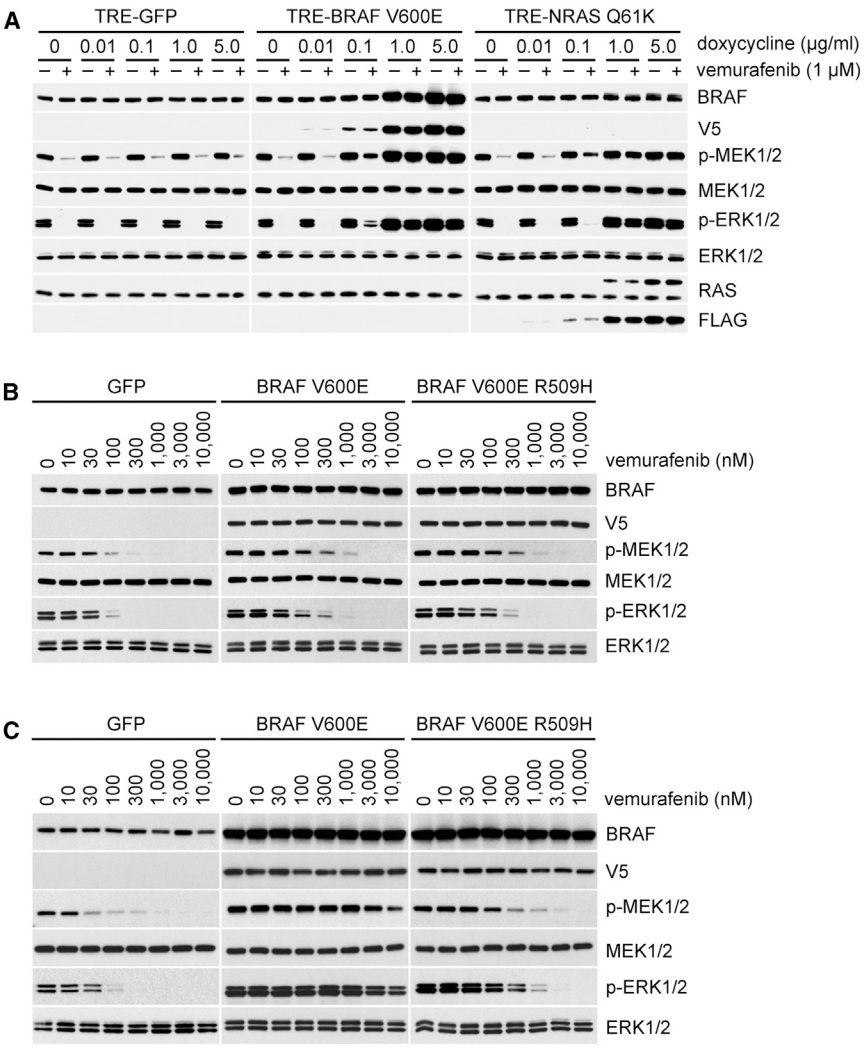


Figure 3. Expression of BRAF V600E Dimers Causes Resistance to Vemurafenib

(A) A375 cells expressing inducible GFP, V5-tagged BRAF V600E, or FLAG-tagged NRAS Q61K were treated with the indicated concentrations of doxycycline for 24 hr, followed by treatment with vemurafenib (1 μM for 1 hr).

(B and C). A375 cells expressing inducible GFP, V5-tagged BRAF V600E, or V5-tagged BRAF V600E R509H were treated with either 0.02 μg/ml (B) or 0.2 μg/ml (C) doxycycline for 24 hr. Cells were then treated with vemurafenib at the indicated doses for 1 hr. Expression of the indicated proteins was assessed by western blot.

See also Figure S3.

and truncated RAFs that lack the N-terminal domain are RAS-independent kinases whose activity is dependent on their constitutive dimerization. A truncated p61 BRAF V600E was found to be responsible for the acquired resistance of some melanomas to RAF inhibitors (Poulikakos et al., 2011). This truncated BRAF V600E is a RAS-independent dimer, but its activity is not abrogated by R509H (Figure 2A). The N-terminal truncation of BRAF V600E causes it to dimerize in a RAS-independent manner, but the V600E mutation causes it to be active as a monomer or dimer. Thus, with the singular exception of BRAF mutants with V600 missense mutations, so far all tested activating mutations, translocations, and fusions of RAF bypass ERK-dependent feedback by constitutively dimerizing in a RAS-independent manner. Uniquely, the V600 mutants do so by functioning as active monomers when RAS-GTP is low.

RAF Mutants that Act as Constitutive Dimers Are Resistant to RAF Inhibitors

The RAF inhibitor vemurafenib inhibits ERK signaling in tumors in which it is driven by BRAF V600E and not in those in which it is driven by RAS (Poulikakos et al., 2010). We used the SKBR3, low RAS-GTP (lapatinib-treated) system to determine the sensitivity to vemurafenib of activating RAF mutants that signal as constitutive dimers. All such mutants were resistant, including non-V600 BRAF mutants, the truncated CRAF (Cat C), and BRAF (p61 WT) proteins (Figure 2C; Table 1) and the two tested fusion proteins (Figure S2D). Vemurafenib transactivates truncated WT dimers, whereas activated BRAF mutants that constitutively dimerize are resistant to the drug but not activated further (Figure 2C). The only BRAF mutants that are sensitive to this drug are the four V600 mutants we tested (Figure 2C; Table 1). Similar results were observed in *Raf1* KO MEFs (Figure S2E). So far there is an absolute correlation between the ability of a mutant to signal as a monomer and its sensitivity to vemurafenib.

Expression of BRAF V600E Dimers Causes Acquired Resistance to Vemurafenib

These results suggest that increased expression of activated RAF dimers will cause resistance to RAF inhibitors. Expression of p61 BRAF V600E or mutant NRAS and *BRAF* V600E amplification are common causes of acquired resistance to these drugs (Nazarian et al., 2010; Poulikakos et al., 2011; Shi et al., 2012). p61 BRAF V600E-dependent acquired resistance depends on its dimerization (Poulikakos et al., 2011), and mutant NRAS promotes the dimerization of BRAF V600E with CRAF (Figure S3A). However, the mechanism whereby *BRAF* V600E amplification induces resistance remains unclear. We used A375, a melanoma cell line homozygous for *BRAF* V600E, to generate stable clones that expressed Tet-regulated BRAF V600E, BRAF V600E R509H, or NRAS Q61K. Increased expression of either BRAF V600E, which is used to model *BRAF* V600E amplification, or mutant NRAS caused increasing resistance to inhibition of ERK signaling by vemurafenib (Figure 3A). Expression of mutant

NRAS was associated with an elevation of cellular RAS-GTP levels (data not shown) and induction of BRAF V600E-CRAF heterodimers (Figure S3A). It is likely that induction of these RAS-driven dimers is responsible for drug resistance.

By contrast, in cells in which BRAF V600E was overexpressed, significant BRAF V600E homodimerization occurs. Increasing amounts of plasmids encoding FLAG-tagged BRAF V600E were transfected into A375 cells that expressed V5-tagged BRAF V600E. As shown in Figure S3B, homodimers were detected when either tag was immunoprecipitated. Homodimer levels increased in direct proportion to the levels of expression of FLAG-tagged BRAF V600E. Under these conditions, neither V600E BRAF/CRAF heterodimerization nor induction of RAS-GTP occurs. These results suggest that *BRAF* V600E amplification causes acquired resistance to RAF inhibitors because it increases BRAF V600E homodimerization. To test this idea, we compared the effects of overexpressing BRAF V600E with those induced by overexpressing the dimerization impaired BRAF V600E R509H. As shown in Figure 3B, cells expressing modest levels of BRAF V600E or BRAF V600E R509H are similarly sensitive to vemurafenib (half-maximal inhibitory concentration [IC_{50}] $\sim$ 100–300 nM). However, at higher levels of expression (Figure 3C), cells with BRAF V600E were significantly less sensitive than those expressing the R509H mutant (IC_{50} > 10,000 versus $\sim$300 nM). Thus, these data suggest that *BRAF* V600E amplification causes acquired resistance by increasing levels of BRAF V600E homodimers.

Binding of RAF Inhibitors to One Site in the Dimer Reduces Their Affinity for the Other

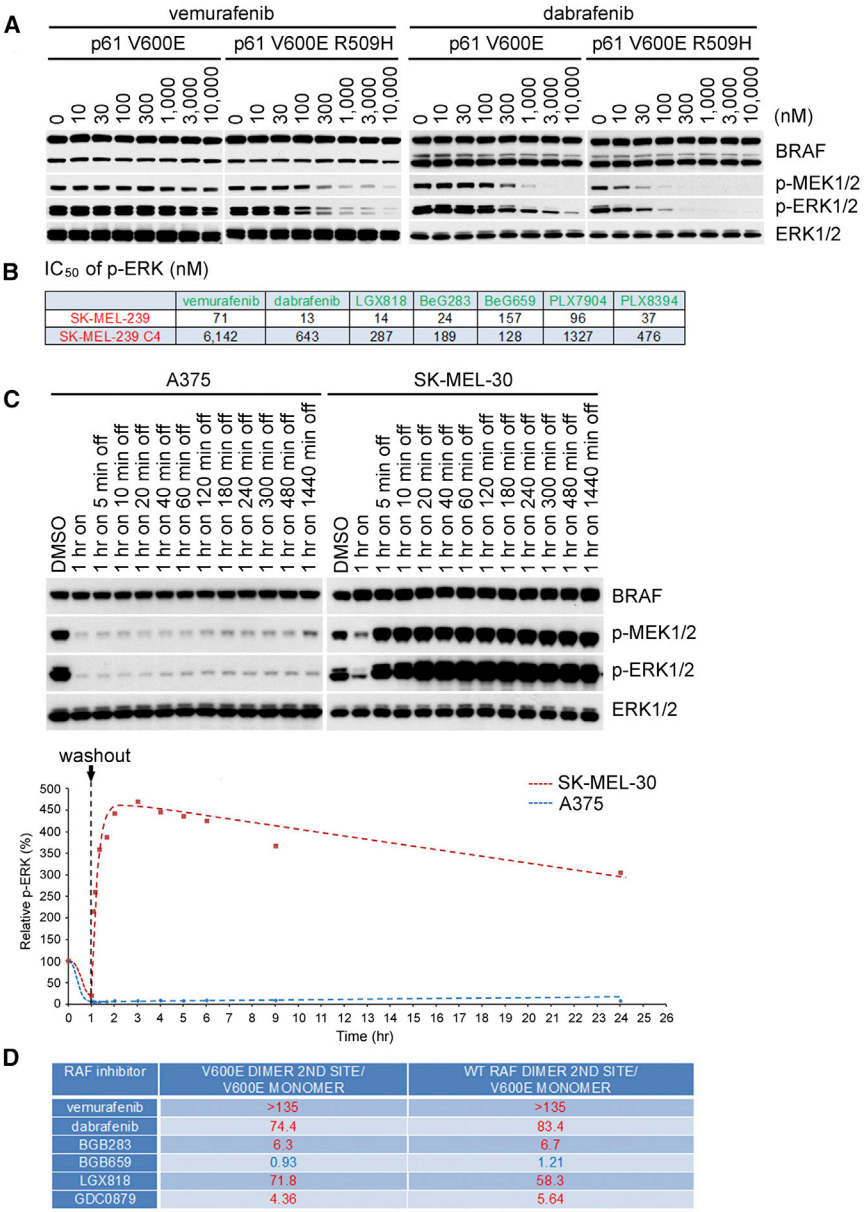
Binding of inhibitors to CRAF or BRAF induces both RAF dimerization and the allosteric transactivation of the unbound protomer of the dimer (Hatzivassiliou et al., 2010; Poulikakos et al., 2010). However, neither mechanism explains why ATP-competitive inhibitors do not bind to both sites and inhibit the dimer. Allosteric regulation of multimeric protein complexes is often associated with cooperative effects on ligand binding. We took advantage of the availability of systems in which the ability of drug to inhibit active monomeric or dimeric mutant BRAF can be compared to assess the possibility that drug binding to one protomer of the dimer reduces its affinity for the other. p61 BRAF V600E signals as a constitutive dimer, whereas p61 BRAF V600E R509H signals as a monomer (Poulikakos et al., 2011). p61 BRAF V600E and p61 BRAF V600E R509H were each expressed in SKBR3, and the effects of RAF inhibitors on ERK signaling were assessed in these cells under low-RAS conditions. Vemurafenib inhibits ERK signaling in SKBR3 cells expressing p61 BRAF V600E R509H or BRAF V600E at similar concentrations (IC_{50} $\sim$100–300 nM) (Figure S4A). We take this as the concentration required to inhibit the monomer. Binding of drug to one protomer of truncated WT dimers causes transactivation of the other. We find that the 50% induction of p61 WT BRAF-driven ERK signaling by vemurafenib also occurs at approximately 100–300 nM (Figures S4B and S4C). This is thus the concentration required to bind to monomers and to the first site of the dimer.

By contrast, more than 30-fold higher concentrations of vemurafenib were required to inhibit ERK driven by p61 V600E dimers than by p61 V600E R509H monomers (Figure 4A) The same relative difference was observed with another, more potent, RAF inhibitor, dabrafenib (Figure 4A). We take the concentration required to inhibit ERK signaling in the p61 BRAF V600E cells as the concentration required inhibiting both sites in the dimer. These data therefore imply that the relative affinity of vemurafenib for the first binding site in a BRAF homodimer is 30-fold higher than that for the second site when the first site is occupied by drug. In isogenic p61 BRAF experiments, the RAF mutants were expressed in a heterologous cellular system: SKBR3. To interrogate a tumor system in which activated BRAF V600E mutant monomers or dimers are expressed, we used SK-MEL239, a BRAF V600E melanoma cell line, and SK-MEL239 C4, a vemurafenib-resistant clone of SK-MEL239 (Poulikakos et al., 2011) that expresses p61 V600E dimers (Figure S4D). Thirty-fold higher concentrations of dabrafenib were required to inhibit ERK signaling in the latter compared with the former (Figure 4B). The data suggests that binding of the drug to the first site in the dimer reduces its affinity for the second and that this explains why monomer-driven ERK signaling is so much more sensitive to these drugs than that driven by dimers.

Identification of a Compound that Inhibits RAF Monomers and Dimers at Similar Concentrations

To identify compounds that inhibit monomers and dimers with similar potency, we screened 22 known RAF inhibitors against SK-MEL-239 and SK-MEL-239 C4. Results for six of these compounds are shown in Figure S4D, and their chemical structures are shown in Table S1. The concentrations required for five of these six to inhibit ERK signaling driven by the dimer were significantly higher (5- to 85-fold) than those that inhibit monomer-driven signaling (Figures 4B and S4D). LGX818 inhibited monomer-driven signaling at 14 nM, and dimer signaling at 287 nM, despite its high potency and low off-rate (see the following discussion). In contrast, BGB659, a type II, ATP-competitive RAF inhibitor (compound 27 from Gould et al., 2011), inhibited ERK signaling driven by p61 BRAF V600E dimers and BRAF V600E monomers at similar doses (Figures 4B and S4D). Its inhibition of ERK signaling is mediated by its binding to BRAF; the T529 BRAF gatekeeper mutation (Heidorn et al., 2010) confers resistance to the drug (Figure S4E). In cells that express either BRAF V600E T529N or p61 BRAF V600E T529N, ERK signaling is resistant to both vemurafenib and BGB659 but not to the MEK inhibitor trametinib.

BGB659 could inhibit BRAF V600E dimers by a variety of mechanisms. We show later (Figure 6B) that, like most RAF inhibitors, BGB659 induces RAF dimerization, so it is not a "dimer breaker." We have not been able to recreate the resistance of RAF dimers to inhibitors in defined in vitro systems. We therefore developed a cellular system in which only one site of the RAF dimer is occupied by a RAF inhibitor and used it to determine the concentration of drug required to bind to and inhibit the second site when the first is already bound. The RAF inhibitor LGX818, which has a very slow off-rate, was used. After 1 hr exposure of BRAF V600E tumor cells to vemurafenib or LGX818, ERK signaling is inhibited (Figure S4F). One hour after washout of the drug, ERK phosphorylation returned to pre-exposure levels in the vemurafenib-treated cell, but remains inhibited in the LGX818-treated cells. Thus, after 1 hr of washout, the BRAF V600E monomer remains bound to LGX818.



	vemurafenib	dabrafenib	LGX818	BeG283	BeG659	PLX7904	PLX8394
SK-MEL-239	71	13	14	24	157	96	37
SK-MEL-239 C4	6,142	643	287	189	128	1327	476

RAF inhibitor	V600E DIMER 2ND SITE/ V600E MONOMER	WT RAF DIMER 2ND SITE/ V600E MONOMER
vemurafenib	>135	>135
dabrafenib	74.4	83.4
BGB283	6.3	6.7
BGB659	0.93	1.21
LGX818	71.8	58.3
GDC0879	4.36	5.64

Figure 4. Identification of an Equipotent Inhibiter of Mutant BRAF Monomers and Dimers

(A) SKBR3 cells expressing p61 V600E or p61 V600E R509H were treated with increasing concentrations of vemurafenib or dabrafenib for 1 hr. Expression of phosphorylated MEK and phosphorylated ERK were assessed by western blot.

(B) IC_{50} values of p-ERK inhibition for a panel of compounds in SK-MEL-239 parental cells (BRAF V600E) or the C4 clone (which expresses p61 BRAF V600E) were calculated on the basis of densitometry analysis of western blot results (n = 3), as shown in Figure S4D. Mean values are listed in the table.

(C) A375 and SK-MEL-30 cells were treated with DMSO or 3 μM LGX818 for 1 hr. Cells were then washed three times with PBS and then placed in drug-free media for the indicated times. p-ERK was assessed by western blot (top) and quantitated by densitometry to generate the dose-response curves using Prism6 (bottom); p-ERK levels relative to those from DMSO-treated cells as a function of time post-treatment are shown in the accompanying graph.

(D) A375, SK-MEL-239 C4, and SK-MEL-30 cells were treated with 1 μM LGX818 for 1 hr, followed by drug washout and treatment with the indicated compounds for an additional 1 hr. The indicated fold changes of p-ERK IC_{50} values were calculated on the basis of the curves, as shown in Figure S4Hd.

See also Figure S4 and Table S1.

SK-MEL-30 is a cell line with mutant NRAS and WT RAF. As expected, vemurafenib activates ERK signaling in these cells by binding to one protomer of WT RAF dimers and transactivating the other (Figure S4G). One hour after washout of vemurafenib, p-ERK returned to baseline, likely because of dissociation of drug from the first site. LGX818 also induced p-ERK at low concentrations, but, in contrast to vemurafenib, induction persisted after 1 hr of washout of the drug, consistent with persistent binding to the first site because of its low off-rate (Figure S4G). Peak induction of ERK occurred at 100 nM LGX818 and actually increased 1 hr after the drug was washed out. At higher concentrations (300–3,000 nM), LGX818 caused a concentration-dependent inhibition of ERK signaling. This is consistent with its binding to and inhibiting both sites in the dimer. At these concentrations, washout for 1 hr hyperactivates ERK signaling compared with untreated controls. This result suggests that the dissociation of drug from the second site is more rapid than that from the first site. The hyperactivation after drug washout is consistent with dissociation of drug from the second site, resulting in accumulation of the half-bound, trans-activated RAF dimer.

This was confirmed when A375 (BRAF V600E) and SK-MEL-30 (NRAS Q61K) cells were treated with 3 μM LGX818 for 1 hr, followed by washout of drug and incubation in normal media (Figure 4C). p-ERK was potently inhibited in both cell lines. After washout, p-ERK remained inhibited in A375 for up to 24 hr, with a slight increase at that time, consistent with the very slow off-rate from the BRAF V600E monomer. After drug was withdrawn from SK-MEL-30, which contains WT RAF dimers, p-ERK rose rapidly, reaching a maximum approximately 1 hr after washout, with a half-time of 10 min. This is consistent with a rapid off-rate of drug bound to the second site in the dimer. In these cells, the maximum p-ERK was about five times higher than basal. After reaching this peak, p-ERK fell slowly, consistent with the slow off-rate of this drug from the first site in the dimer, and was still 3-fold elevated compared with basal 24 hr after washout (Figure 4C).

Thus, after exposure of cells to high concentrations of LGX818, washout of the drug for 1 hr leads to accumulation of activated half-bound dimers. We reasoned that the

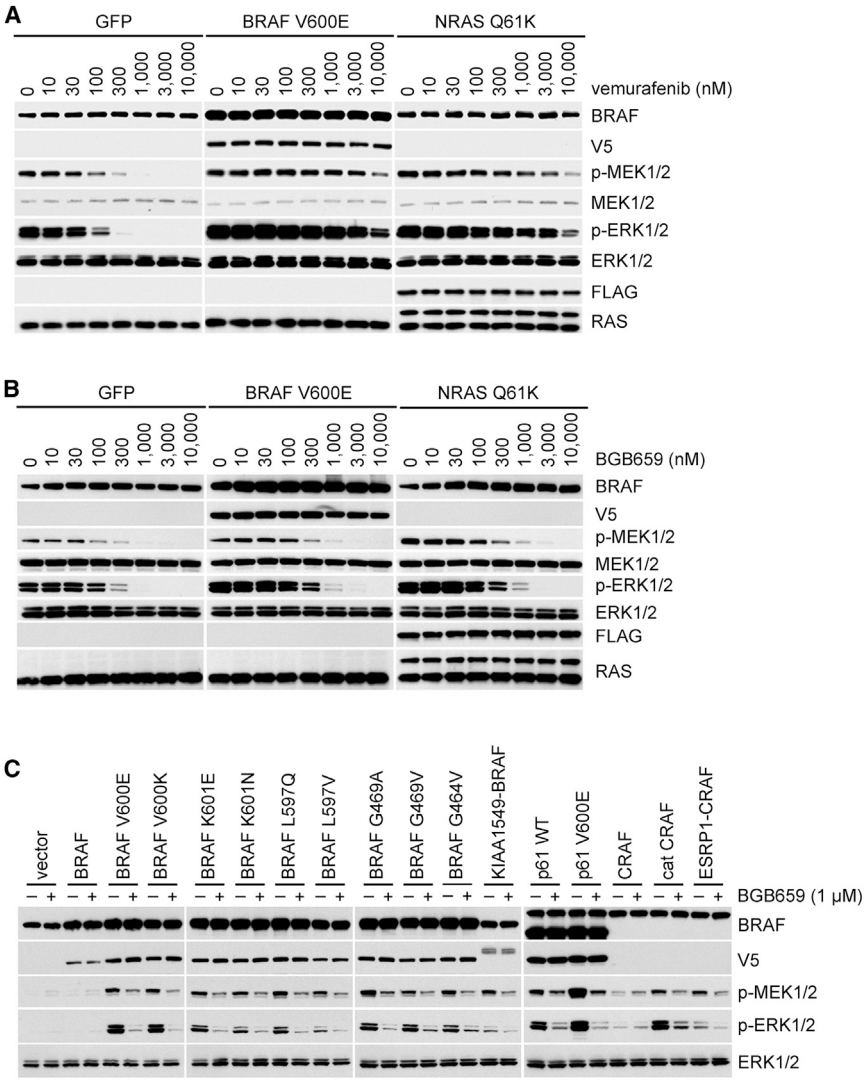


Figure 5. BGB659 Effectively Inhibits Vemurafenib-Resistant ERK Signaling

(A and B) A375 cells expressing inducible GFP (control), BRAF V600E, or NRAS Q61K were treated with doxycycline (2 μg/ml for 24 hr), followed by treatment with vemurafenib (A) or BGB659 (B) at the indicated concentrations. Cell lysates were then analyzed by western blot using the antibodies indicated.

(C) SKBR3 cells were transfected with plasmids encoding the indicated WT or mutant proteins. After 24 hr, cells were treated with BGB659 (1 μM for 1 hr). Expression and/or phosphorylation of the indicated proteins was assayed by western blot. See also Figure S5.

concentration of other RAF inhibitors that is required to inhibit ERK signaling in these cells 1 hr after LGX818 washout reflects the relative affinity of the drug for the second (unoccupied) site.

We used this system to determine the concentrations of six RAF inhibitors that are required to bind the second site of the dimer when the first is occupied by drug. For each inhibitor, this value was compared with the concentration at which it inhibits BRAF V600E monomers. Five of the six compounds inhibited ERK phosphorylation in A375 BRAF V600E melanoma cells at concentrations ranging from 10–100 nM (Figure S4H-a). This is the concentration required to inhibit the monomer (or the first site in the dimer). To determine the concentration of these drugs required inhibit the second site when the first is bound to drug, SK-MEL-30 NRAS mutant cells were exposed to 1 μM LGX818 for 1 hr, after which it was washed out for 1 hr. Other RAF inhibitors were then added for 1 hr to assess the concentration at which they inhibit ERK phosphorylation. All the drugs were able to inhibit p-ERK but, for five of the six, at concentrations much greater than those at which they inhibit the monomer (0.3–10 μM) (Figure S4H-b versus Figure S4H-a). Only BGB659 inhibited the monomer and the second site of the WT RAF dimer at approximately the same concentrations. Very similar results were obtained when the same experiment was done in SK-MEL-239 C4 with BRAF V600E dimers (p61 BRAF V600E) (Figure S4H-c). These results are shown graphically (Figure S4H-d) and as ratios of concentrations required to inhibit the second site compared with those that inhibit the monomer (Figure 4D).

These data suggest that binding of most RAF inhibitors to one site in WT or mutant RAF dimers substantially reduces their affinity for the second site and that this accounts for the resistance of dimer-driven ERK signaling to these drugs. However, the binding of BGB659 is unaffected by occupancy of the first site, and it inhibits monomers and the second site of dimers with similar potency (100–300 nM). Our model suggests that such a drug would be effective in treating tumors in which oncogenic ERK signaling is driven by RAF dimers.

BGB659 Effectively Inhibits ERK Signaling Driven by Oncogenic BRAF Dimers in Tumor Cells

In A375, expression of mutant NRAS or overexpression of BRAF V600E caused ERK signaling to become much less sensitive to RAF monomer selective inhibitors (Figures 5A and S5A). By contrast, the concentration at which BGB659 inhibits ERK signaling was affected only marginally by BRAF V600E overexpression or mutant NRAS expression (Figure 5B), and it inhibits ERK signaling in BRAF V600E melanoma SK-MEL-239 and its resistant counterpart SK-MEL-239 C4 at similar concentrations (Figure 4B). The effects of these drugs on cell proliferation are closely correlated with their effects on ERK signaling. The concentrations of BGB659 that cause inhibition of the proliferation of A375, A375-expressing mutant NRAS, or A375-overexpressing BRAF V600E are very similar (Figure S5B). Significantly higher concentrations of the other tested inhibitors were required to inhibit the proliferation of melanoma cells with any of the three

mechanisms of dimer-dependent resistance (Figures S5B and S5C). Thus, BGB659 effectively inhibits the growth of melanoma cells in which acquired resistance to vemurafenib is driven by truncated or full-length BRAF V600E dimers.

BGB659 also inhibited ERK signaling driven by RAF mutants that constitutively dimerize. All such mutants are resistant to inhibition by 1 μM vemurafenib (Figure 2C; Table 1) but sensitive to 1 μM BGB659 (7 of 10 mutants tested are shown in Figure 5C). BGB659 also inhibits ERK signaling driven by activated RAF fusion proteins (KIA1549-BRAF and ESRP1-CRAF) and truncations (p61 or Cat C) that constitutively dimerize (Figure 5C). BGB659 effectively inhibits ERK signaling and the proliferation of JVM-3, a BRAF K601N CLL cell line that is resistant to vemurafenib (Figures S5D and S5E). Thus, BGB659 inhibits ERK signaling driven by both oncogenic RAF monomers and dimers and inhibits the proliferation of tumor cells harboring these RAF mutants.

BGB659 Preferentially Inhibits Mutant RAF-Driven ERK Signaling

An inhibitor of mutant and WT RAF dimers would inhibit ERK in normal cells and would have a narrow therapeutic index, as do MEK inhibitors. We compared the effects of BGB659 on signaling driven by WT dimers and mutant RAF dimers in NIH 3T3 cells in which a variety of RAS or RAF mutants were expressed in an inducible manner. The IC_{50} values for BGB659 inhibition of ERK signaling driven by BRAF V600E monomers or three different mutant BRAF constitutive dimers were very similar and more than an order of magnitude lower than those required to inhibit signaling in cells in which WT BRAF or NRAS or NRAS Q61K were overexpressed (Figure S6A). The data suggest that BGB659 does not inhibit all RAF dimers equally; BRAF mutant monomers and dimers are more sensitive than RAS-driven WT RAF dimers. In contrast, after 1 hr of exposure, the MEK inhibitor trametinib inhibits ERK phosphorylation at similar doses whether it is driven by WT RAF or mutant BRAF (Figure S6B).

This was also the case in tumor cell lines in which ERK is activated by different upstream mechanisms (Figure 6A), including mutant BRAF V600E (A375 melanoma), A375 in which NRAS Q61K is expressed, mutant NRAS Q61R (SK-MEL-2 melanoma), mutant KRAS G12S (A549 lung cancer), and HER2-activated WT RAS (SKBR3 breast cancer). BGB659 inhibits ERK signaling at almost identical concentrations in A375 and in A375 in which mutant NRAS has been overexpressed (IC_{50} ~100–300 nM, almost complete inhibition at 1 μM). In contrast, in the other three cell lines, ERK phosphorylation increased after exposure to relatively low doses of the drug, with maxima occurring at 50–100 nM, and declined at higher concentrations. In SKBR3 and SK-MEL-2, ERK phosphorylation declined 40% with 1 μM BGB659 and only approached complete inhibition at 10 μM. In A549 mutant KRAS cells, ERK declined to pretreatment levels at 1 μM drug, and some residual activity remained at 10 μM. Thus, tumor cells with mutant RAF, with or without coexistent RAS mutation, are most sensitive to BGB659. In cells in which ERK is driven by WT RAF (SK-MEL-2, A549, SKBR3), the effect of the drug varied with concentration in a biphasic manner, with ERK phosphorylation enhanced at low doses and inhibited at higher doses. The effects of BGB659 on tumor cell proliferation correlated with its effects on signaling (Figure S6C). All 15 tumor cell lines with mutant BRAF were sensitive to the drug (9 with BRAF V600E, 1 with BRAF V600K, 4 with BRAF mutants that constitutively dimerize, and 1 with p61 BRAF V600E) with IC_{50} values from 100 to 600 nM. By contrast, the 10 tumor cell lines with RAS mutation and 14 with WT RAS and RAF were 10- to more than 50-fold less sensitive than the mutant RAF tumors.

A biphasic dose response is also observed when normal or tumor cells with activated RAS are treated with previously reported RAF inhibitors (Poulikakos et al., 2010). This phenomenon has been shown to result from both induction of RAF dimerization by the drug and its transactivation of RAF dimers (Hatzivassiliou et al., 2010; Lavoie et al., 2013; Poulikakos et al., 2010). BGB659 inhibits the second site of activated RAF dimers in cells with mutant NRAS with the same potency as it inhibits BRAF V600E monomers (Figures S4Ha and S4Hb). We therefore asked whether its differential effects on mutant and WT RAF dimers are due to differential induction of dimerization. BGB659 and other RAF inhibitors caused marked, dose-dependent induction of BRAF/CRAF heterodimers in HeLa and in two melanoma cell lines with mutant NRAS, but not in A375 (BRAF V600E) (Figure 6B). Induction by BGB659, and the RAF monomer inhibitors LGX 818 and dabrafenib was equivalent and much greater than that caused by vemurafenib (Figure 6B). Because physiologic dimerization of RAF is RAS dependent and RAS-GTP levels are inhibited by feedback in A375, we asked whether induction of dimerization is RAS dependent. In SKBR3 cells, RAS-GTP levels and induction of BRAF/CRAF heterodimers by BGB659 were both inhibited in a dose-dependent fashion by lapatinib and correlated closely with each other (Figure 6C). These data suggest that induction of dimerization is RAS dependent and does not occur in cells with activating RAF mutations because their RAS-GTP levels are low.

In support of this model, we examined the ability of BGB659 to induce dimerization of WT RAF in cells with active RAS-GTP (Figure 6D, lanes 1–6). In SKBR3, BGB659 induces WT BRAF homodimers and BRAF/CRAF heterodimers (Figure 6D, lane 4 versus 3, lane 6 versus 5). Thus, in cells in which WT RAFs are activated by active RAS, BGB659 both induces the formation of active RAF dimers and inhibits their activity.

In contrast, in cells that express activated RAF mutants, RAS is feedback inhibited, and ERK signaling is RAS independent. Accordingly, we assessed the effects of BGB659 on RAF dimerization in lapatinib-treated SKBR3 cells engineered to express different RAF mutants. (Figure 6D, lanes 7–12). In BRAF mutant-expressing cells, no mutant BRAF or WT CRAF heterodimers are detected. Significant levels of p61 BRAF (Figure 6D, lanes 7 and 8) and BRAF K601E (Figure 6D, lanes 11 and 12) homodimers are expressed in cells in which either mutant is expressed. In cells in which BRAF V600E is expressed, a low level of mutant homodimers is observed (Figure 6D, lanes 9 and 10). BGB659 did not induce mutant RAF homo- or heterodimerization in any of these cells. Thus, BGB659 induces RAS-dependent dimerization of WT RAF, but in tumors with mutant RAF, RAS-GTP levels are too low to support induction of dimerization. Taken together, these data support the following model. BGB659 inhibits both sites of RAF dimers and also induces RAS-dependent RAF dimerization. In cells with adequate levels of RAS activation, this accounts for the biphasic response to

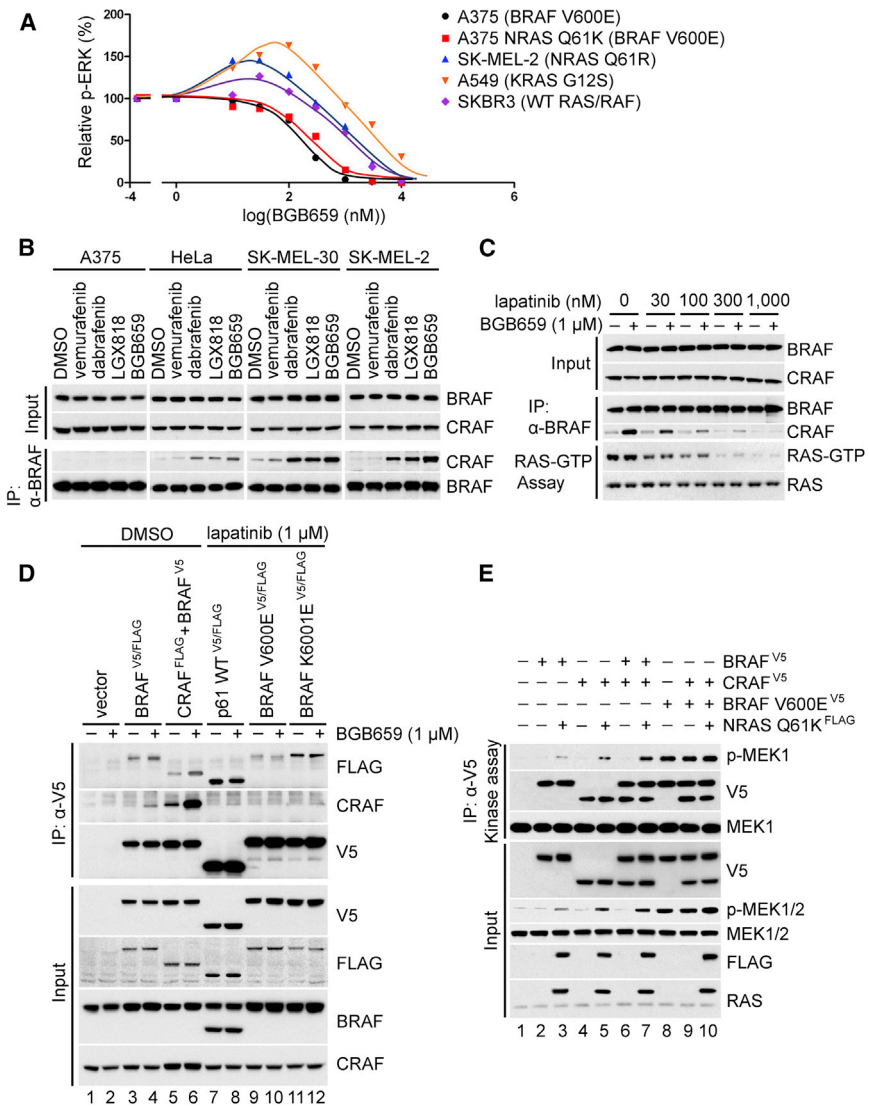


Figure 6. BGB659 Preferentially Inhibits Signaling Driven by Mutant BRAF Dimers

(A) The indicated cell lines were treated with 0, 10, 30, 100, 300, 1,000, 3,000, and 10,000 nM BGB659 for 1 hr. Whole-cell lysates were assayed by western blot with anti-p-ERK antibody. The p-ERK level of each sample was quantitated by densitometry and then normalized to the p-ERK level in untreated cells. The p-ERK response curves were generated using Prism6.

(B) A375, HeLa, SK-MEL-30, and SK-MEL-2 cells were treated with the indicated compounds for 1 hr. Endogenous BRAF was immunoprecipitated with anti-BRAF antibody. The input and isolated protein complexes were assayed by western blot as indicated.

(C) SKBR3 cells were pre-treated with lapatinib at the indicated concentrations for 1 hr, followed by treatment with 1 μM BGB659 or vehicle. Cell lysates were then subjected to immunoprecipitation with anti-BRAF antibody or the RAS-GTP pull-down assay. Binding of CRAF to BRAF and RAS-GTP levels were determined by western blot. Expression levels of BRAF and CRAF were determined using whole-cell lysates.

(D) The indicated RAF proteins were expressed in SKBR3 cells for 24 hr. Cells were then treated with either 1 μM lapatinib or the equivalent volume of DMSO for 1 hr, followed by treatment with 1 μM BGB659 or DMSO for an additional 1 hr. All cell lysates were collected and subjected to immunoprecipitation with an anti-V5 antibody. The isolated protein complexes and input were analyzed by western blot.

(E) BRAF-V5, CRAF-V5, BRAF V600E-V5, and FLAG-tagged NRAS Q61K were transiently expressed in SKBR3 cells, followed by 1 hr treatment with 1 μM lapatinib. The cells were then collected and lysed, and the ectopically expressed RAF proteins were isolated with anti-V5 beads, followed by elution with V5 peptide. The kinase activity of isolated RAF kinases was determined by in vitro kinase assay with K97R MEK1 as substrate. The indicated proteins from both input and kinase assays were assayed by western blot.

See also Figure S6.

increasing concentrations of BGB659. In contrast, in cells with activating RAF mutants, RAS-GTP is low, and the drug inhibits the activity of RAF dimers without inducing their formation. Tumors with RAF mutants are thus more sensitive to this drug than those with mutant RAS or normal cells.

The exceptional case that complicates this model is the BRAF V600E tumor with acquired resistance due to mutant NRAS. These tumors are as sensitive to BGB659 as those with BRAF V600E alone. We asked whether BGB659 affects RAF dimerization in cells with co-expression of BRAF V600E and mutant NRAS (Figure S6D). BRAF homodimers and BRAF/CRAF heterodimers are barely detectable in SKBR3 treated with lapatinib. Co-expression of mutant NRAS with WT RAF significantly increases levels of homo- and heterodimers and BGB659 further enhances expression of these dimers (Figure S6D, lanes 2–4). When BRAF V600E and WT CRAF are overexpressed in SKBR3, BRAF V600E homodimers and heterodimers were barely detectable. These dimers were markedly enhanced when mutant NRAS was co-expressed and further enhanced by BGB659 (Figure S6D, lanes 6–8). Thus, the drug can induce BRAF V600E dimerization in tumors that co-express mutant RAS. However, in this case, induction of BRAF V600E dimerization is not associated with a significant induction of RAF kinase activity (Figure 6E). Co-expression of mutant NRAS induces BRAF V600E dimerization (Figure S6D); it has almost no effect on elevated RAF kinase activity (Figure 6E, lanes 8–10). This is consistent with our data that RAF inhibitors do not paradoxically activate p-ERK in such tumors. By contrast, co-expression of WT BRAF or CRAF with mutant NRAS is associated with increased RAF kinase activity (Figure 6E, lanes 2–6). Thus, BGB659 effectively inhibits ERK signaling in tumors that co-express BRAF V600E and mutant RAS because in these cells, it induces BRAF V600E dimerization, but not kinase activation. BGB659 works less well in cells with active RAS and WT RAF, because it increases RAF kinase activity by inducing dimerization.

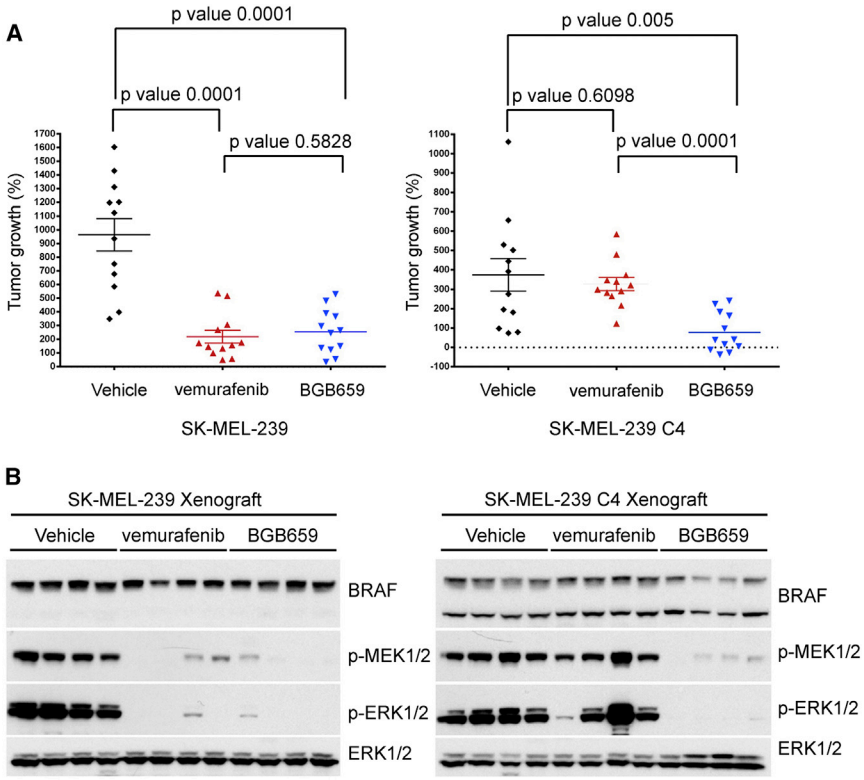


Figure 7. BGB659 Inhibits the In Vivo Growth of BRAF V600E Tumors with Acquired Resistance to Vemurafenib

(A and B) SK-MEL-239 parental or C4 clone (p61 V600E) cells were injected subcutaneously into the opposite flanks of nude mice (1.5×10^7 cells per injection). After 10 days, all 12 tumors from each group were 100–150 mm^3 in size, and the indicated drug treatments were started. The graph shows the size of each tumor after 32 days of daily treatment (A) (n = 12, error bars indicate mean ± SEM, p values were calculated using the unpaired t test). Protein extracts from 4 randomly selected tumors from each group were analyzed by western blot using the antibodies indicated (B).

BGB659 Inhibits the In Vivo Growth of Tumors Driven by Mutant RAF Monomers or Dimers

Figures 6 and S6 suggest that BGB659 will inhibit ERK signaling in tumors driven by activating RAF mutants and fusion proteins more effectively than in normal cells and could therefore be useful clinically. We tested whether BGB659 could inhibit the in vivo growth of SK-MEL-239 C4, cells in which acquired resistance to vemurafenib is mediated by the p61 BRAF V600E dimer. This model and the vemurafenib-sensitive parental SK-MEL-239 cell line were grown as subcutaneous murine xenografts. BGB659 (100 mg/kg) given daily was unassociated with weight loss or other obvious toxicity. Mice carrying 100 mm^3 tumors were treated daily for 32 days with vehicle, 75 mg/kg vemurafenib, or 100 mg/kg BGB659 and then analyzed. Vemurafenib and BGB659 both effectively inhibited the growth of SK-MEL-239 tumors, but only the latter had activity against SK-MEL-239 C4 tumors (Figure 7A). Consistently, vemurafenib and BGB659 both potently inhibited ERK signaling in SK-MEL-239 tumors, but only BGB659 inhibited ERK signaling in SK-MEL-239 C4 tumors (Figure 7B). These results support the possibility that drugs of this class could be effective in tumors driven by either mutant RAF monomers or dimers, including those that mediate acquired resistance to current RAF inhibitors.

DISCUSSION

Almost 200 BRAF mutants and many RAF translocations have been identified in human cancer, and many of those have increased catalytic activity (Wan et al., 2004; Palanisamy et al., 2010). Our work divides the activating mutants into two functional classes on the basis of the mechanism whereby they achieve RAS independence. The dimerization of BRAF V600 mutants remains RAS dependent, but they also signal as RAS-independent monomers in tumor cells in which RAS is inhibited by feedback. All of the other tested RAF mutants and fusion proteins signal as RAS-independent constitutive dimers. These data support the idea that insensitivity to physiologic feedback is a common property of oncoproteins that is required for their hyperactivation of signaling output.

None of the RAF mutants that signal as constitutive dimers are sensitive to the previously reported RAF inhibitor vemurafenib. This drug preferentially inhibits active RAF monomers, which comprise all four of the BRAF V600 mutant alleles found in patients. These mutants are capable of signaling as monomers or RAS-dependent dimers; the feedback inhibition of RAS by ERK causes BRAF V600 mutants to exist as drug-sensitive monomers in these tumors. Moreover, the three most common causes of acquired resistance of BRAF V600E melanomas to RAF inhibitors—NRAS mutation, splicing of BRAF V600E that produces a truncated BRAF kinase, and BRAF V600E overexpression due to gene amplification—all cause resistance by causing dimerization of BRAF V600E.

The mechanisms underlying the characteristic effects of RAF inhibitors, inhibition of BRAF V600E monomers, resistance of RAF dimers, and activation of ERK signaling in cells with active RAS and WT RAF remain controversial. The designation of these drugs as selective inhibitors of BRAF persists in the medical literature despite evidence to the contrary. They have been shown to inhibit the kinase activities of all three RAF family members in vitro and to activate ERK signaling in BRAF KO cells with active RAS (Poulikakos et al., 2010). We show here that BRAF mutants that constitutively dimerize are resistant to these drugs, as are V600E dimers in cells with coexistent NRAS mutation or overexpressing V600E. These drugs would be better described as inhibitors of BRAF monomers. However, why these drugs are poor inhibitors of dimers is poorly understood. Binding of RAF inhibitors to one protomer in the WT CRAF dimer

causes the allosteric transactivation of the unbound protomer. Most inhibitors also cause RAF to dimerize. Both of these effects play a role in transactivation but cannot, by themselves, explain paradoxical activation of WT dimers or the insensitivity of mutant dimers, because, even if binding of the inhibitor to one protomer causes the other to adopt an active conformation, the drug ought to bind to the second site in the dimer with similar potency.

The obvious hypothesis is that binding of the drug to one site in the dimer causes an allosteric effect that reduces its affinity for the second site. We now provide evidence that this is the case. In isogenic models, the concentrations of RAF inhibitor required to inhibit the monomer were much lower than those required to inhibit the dimer. We take these findings as reflecting the difference in concentration required to inhibit the first and second sites of the dimer and therefore demonstrating that negative cooperativity is induced on occupancy of the first site by the drug. This idea was supported by experiments in which the off-rate of a RAF inhibitor from the first and second sites of an active WT dimer were measured in cells, and the latter was found to be considerably faster than the former. This system was used to measure the concentrations at which several RAF inhibitors inhibit the second site when the first was occupied by the low off-rate drug. In all cases, the concentration at which the standard RAF inhibitors block signaling driven by the second site is considerably higher than the concentration required for them to inhibit BRAF V600E monomers. Taken together, these data suggest that significantly higher concentrations of RAF inhibitors are required to inhibit dimers than monomers because occupancy of the first site in the dimer by the drug reduces its affinity for the second site.

Our data suggest that an inhibitor of RAF dimers could be useful for the treatment of many types of ERK-dependent tumors. We identified BGB659 that inhibits BRAF dimers at about the same concentration as monomers. Moreover, binding of BGB659 to RAF dimers is unaffected by induction of negative cooperativity by drug occupancy of the first site. As predicted, BGB659 inhibits ERK signaling driven by both active monomers and constitutively activated dimers. It also inhibits ERK signaling in models in which acquired resistance to standard RAF inhibitors is mediated by mutant BRAF dimers. However, because it also induces the formation of activated WT RAF dimers in cells with active RAS, it is a much less potent inhibitor of ERK signaling in normal cells and in tumors with RAS mutation.

This work has several important clinical implications. First, it suggests that current RAF inhibitors will be effective in tumors driven by BRAF V600 mutants and not in those driven by any of the other, constitutively dimerizing, BRAF mutants or translocations. This must be a tentative conclusion, because we do not understand the structural basis whereby all of these mutants (including K601E) form constitutive RAS-independent dimers, and only BRAF V600 mutants can signal as either dimers or monomers. Crystal structures have not shed light on this question, perhaps because they all lack the amino-terminal portion of RAF proteins that is critical in regulating dimerization. We are therefore engaged in characterizing the RAS and dimerization dependence of all mutant RAF alleles found in tumors and determining their sensitivity to inhibitors.

Second, we describe herein an algorithm for assessing the mechanism of action and drug sensitivity of uncharacterized or newly identified mutations and translocations.

Third, BGB659 inhibits ERK signaling driven by mutant RAF monomers and dimers at doses at which it does not inhibit signaling in normal cells. These data suggest that this type of drug will have a wide therapeutic index and could be effective in tumors in which current RAF inhibitors are ineffective: tumors driven by non-V600E BRAF mutants, activating RAF dimers encoded by gene translocations, and BRAF V600E tumors in which acquired resistance to RAF inhibitor is due to dimerization. It is also possible that such a drug will be superior to currently available RAF inhibitors as an initial treatment to the tumors driven by BRAF V600E, because they will not be subject to many of the most common mechanisms of acquired resistance to those drugs. With the widespread sequencing of human tumor tissue, many such fusion and non-BRAF V600-activating mutants are being discovered, and such drugs ought to have wide clinical utility.

EXPERIMENTAL PROCEDURES

Compounds

BGB283 and BGB659 were obtained from BeiGene. Vemurafenib, PLX7904, and PLX8394 were obtained from Plexxikon. Lapatinib, trametinib, and dabrafenib were obtained from GlaxoSmithKline. LGX818 was obtained from from Novartis. LY3009120 was purchased from Active Biochem; doxycycline from Sigma Aldrich; puromycin and hygromycin stock solution from Invitrogen; and other drugs from Selleckchem. Drugs were dissolved in DMSO to yield 10 mM stock and stored at −20°C.

Cell Culture

All cell lines were obtained from either the Memorial Sloan-Kettering Cancer Center (MSKCC) cell collection or the American Type Culture Collection, except the conditional RAS KO cell line, which was provided by Mariano Barbacid. 22RV1, H1395, OCI/AML3, U266, JVM-3, and SIG-M5 were cultured in RPMI + 10% fetal bovine serum (FBS). Keratinocytes were maintained in the Defined K-SFM medium from GIBCO. All other cell lines were grown in Dulbecco's modified Eagle's medium with glutamine, antibiotics, and 10% FBS. The inducible expression cells were maintained in the medium with 50 μg/ml hygromycin and 0.2 μg/ml puromycin.

Antibodies

Western blot, immunoprecipitation, and in vitro kinase assays were performed as described (Poulikakos et al., 2011). The following antibodies were used: anti-p217/p221-MEK1/2 (p-MEK1/2), anti-p202/p204-ERK1/2 (p-ERK1/2), anti-MEK1/2, anti-ERK1/2 from Cell Signaling, anti-V5 from Invitrogen, anti-BRAF from Santa Cruz Biotechnology, anti-FLAG from Sigma, anti-CRAF from BD Transduction Laboratories, and anti-CRAF-S338 from Millipore. For immunoprecipitations of tagged proteins, the following reagents were used: anti-V5 agarose affinity gel (Invitrogen), anti-Flag M2 affinity gel (Sigma), and protein G agarose gel (Invitrogen).

Plasmids

The pcDNA3-BRAF-V5/FLAG/myc, pcDNA3-CRAF-V5/FLAG/myc, pcDNA3-catC-FLAG, and pcDNA3-p61/p61 R509H were constructed as previously described (Poulikakos et al., 2010, 2011). The ESRP1-RAF1 and KIAA1549-BRAF fusion genes were sub-cloned into pcDNA3 with FLAG or V5 tag. Plasmids for retroviral-based inducible expression system were provided by Scott Lowe's lab at MSKCC. The BRAF and NRAS genes were sub-cloned into TTIGFP-MLUEX vector harboring tet-regulated promoter. Mutations were introduced by using the site-directed Mutagenesis Kit (Stratagene).

Animal Model Studies

Nu/nu athymic mice were obtained from Harlan Laboratories and maintained in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines. Subcutaneous xenografts and tumor measurements were performed as described. All studies were performed in compliance with institutional guidelines under an IACUC-approved protocol.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ccell.2015.08.001.

AUTHOR CONTRIBUTIONS

Z.Y. and N.R. conceived the hypotheses, designed and analyzed the experiments, and wrote the manuscript. Z.Y., N.M.T., A.T., Y.G., Q.L., and E.S. performed and analyzed the experiments. O.A.-W., L.L., D.B.S., and P.I.P. contributed to experimental design and data analysis.

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REFERENCES

Avraham, R., and Yarden, Y. (2011). Feedback regulation of EGFR signalling: decision making by early and delayed loops. Nat. Rev. Mol. Cell Biol. *12*, 104–117.

Berghoff, A.S., and Preusser, M. (2014). BRAF alterations in brain tumours: molecular pathology and therapeutic opportunities. Curr. Opin. Neurol. *27*, 689–696.

Corcoran, R.B., Ebi, H., Turke, A.B., Coffee, E.M., Nishino, M., Cogdill, A.P., Brown, R.D., Della Pelle, P., Dias-Santagata, D., Hung, K.E., et al. (2012). EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib. Cancer Discov. *2*, 227–235.

Cutler, R.E., Jr., Stephens, R.M., Saracino, M.R., and Morrison, D.K. (1998). Autoregulation of the Raf-1 serine/threonine kinase. Proc. Natl. Acad. Sci. U S A *95*, 9214–9219.

Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W., et al. (2002). Mutations of the BRAF gene in human cancer. Nature *417*, 949–954.

Dong, C., Waters, S.B., Holt, K.H., and Pessin, J.E. (1996). SOS phosphorylation and disassociation of the Grb2-SOS complex by the ERK and JNK signaling pathways. J. Biol. Chem. *271*, 6328–6332.

Dougherty, M.K., Müller, J., Ritt, D.A., Zhou, M., Zhou, X.Z., Copeland, T.D., Conrads, T.P., Veenstra, T.D., Lu, K.P., and Morrison, D.K. (2005). Regulation of Raf-1 by direct feedback phosphorylation. Mol. Cell *17*, 215–224.

Drosten, M., Sum, E.Y., Lechuga, C.G., Simón-Carrasco, L., Jacob, H.K., García-Medina, R., Huang, S., Beijersbergen, R.L., Bernards, R., and Barbacid, M. (2014). Loss of p53 induces cell proliferation via Ras-independent activation of the Raf/Mek/Erk signaling pathway. Proc. Natl. Acad. Sci. U S A *111*, 15155–15160.

Fabian, J.R., Vojtek, A.B., Cooper, J.A., and Morrison, D.K. (1994). A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. Proc. Natl. Acad. Sci. U S A *91*, 5982–5986.

Freeman, A.K., Ritt, D.A., and Morrison, D.K. (2013). Effects of Raf dimerization and its inhibition on normal and disease-associated Raf signaling. Mol. Cell *49*, 751–758.

Gould, A.E., Adams, R., Adhikari, S., Aertgeerts, K., Afroze, R., Blackburn, C., Calderwood, E.F., Chau, R., Chouitar, J., Duffey, M.O., et al. (2011). Design and optimization of potent and orally bioavailable tetrahydronaphthalene Raf inhibitors. J. Med. Chem. *54*, 1836–1846.

Hatzivassiliou, G., Song, K., Yen, I., Brandhuber, B.J., Anderson, D.J., Alvarado, R., Ludlam, M.J., Stokoe, D., Gloor, S.L., Vigers, G., et al. (2010). RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature *464*, 431–435.

Heidorn, S.J., Milagre, C., Whittaker, S., Nourry, A., Niculescu-Duvas, I., Dhomen, N., Hussain, J., Reis-Filho, J.S., Springer, C.J., Pritchard, C., and Marais, R. (2010). Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell *140*, 209–221.

Lang, R., Hammer, M., and Mages, J. (2006). DUSP meet immunology: dual specificity MAPK phosphatases in control of the inflammatory response. J. Immunol. *177*, 7497–7504.

Lavoie, H., Thevakumaran, N., Gavory, G., Li, J.J., Padeganeh, A., Guiral, S., Duchaine, J., Mao, D.Y., Bouvier, M., Sicheri, F., and Therrien, M. (2013). Inhibitors that stabilize a closed RAF kinase domain conformation induce dimerization. Nat. Chem. Biol. *9*, 428–436.

Lito, P., Pratilas, C.A., Joseph, E.W., Tadi, M., Halilovic, E., Zubrowski, M., Huang, A., Wong, W.L., Callahan, M.K., Merghoub, T., et al. (2012). Relief of profound feedback inhibition of mitogenic signaling by RAF inhibitors attenuates their activity in BRAFV600E melanomas. Cancer Cell *22*, 668–682.

Luo, Z., Tzivion, G., Belshaw, P.J., Vavvas, D., Marshall, M., and Avruch, J. (1996). Oligomerization activates c-Raf-1 through a Ras-dependent mechanism. Nature *383*, 181–185.

Mason, C.S., Springer, C.J., Cooper, R.G., Superti-Furga, G., Marshall, C.J., and Marais, R. (1999). Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. EMBO J. *18*, 2137–2148.

Montero-Conde, C., Ruiz-Llorente, S., Dominguez, J.M., Knauf, J.A., Viale, A., Sherman, E.J., Ryder, M., Ghossein, R.A., Rosen, N., and Fagin, J.A. (2013). Relief of feedback inhibition of HER3 transcription by RAF and MEK inhibitors attenuates their antitumor effects in BRAF-mutant thyroid carcinomas. Cancer Discov. *3*, 520–533.

Nazarian, R., Shi, H., Wang, Q., Kong, X., Koya, R.C., Lee, H., Chen, Z., Lee, M.K., Attar, N., Sazegar, H., et al. (2010). Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. Nature *468*, 973–977.

Paik, P.K., Arcila, M.E., Fara, M., Sima, C.S., Miller, V.A., Kris, M.G., Ladanyi, M., and Riely, G.J. (2011). Clinical characteristics of patients with lung adenocarcinomas harboring BRAF mutations. J. Clin. Oncol. *29*, 2046–2051.

Palanisamy, N., Ateeq, B., Kalyana-Sundaram, S., Pflueger, D., Ramnarayanan, K., Shankar, S., Han, B., Cao, Q., Cao, X., Suleman, K., et al. (2010). Rearrangements of the RAF kinase pathway in prostate cancer, gastric cancer and melanoma. Nat. Med. *16*, 793–798.

Poulikakos, P.I., Zhang, C., Bollag, G., Shokat, K.M., and Rosen, N. (2010). RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. Nature *464*, 427–430.

Poulikakos, P.I., Persaud, Y., Janakiraman, M., Kong, X., Ng, C., Moriceau, G., Shi, H., Atefi, M., Titz, B., Gabay, M.T., et al. (2011). RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). Nature *480*, 387–390.

Pratilas, C.A., Taylor, B.S., Ye, Q., Viale, A., Sander, C., Solit, D.B., and Rosen, N. (2009). (V600E)BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway. Proc. Natl. Acad. Sci. U S A *106*, 4519–4524.

Rajakulendran, T., Sahmi, M., Lefrançois, M., Sicheri, F., and Therrien, M. (2009). A dimerization-dependent mechanism drives RAF catalytic activation. Nature *461*, 542–545.

Shi, H., Moriceau, G., Kong, X., Lee, M.K., Lee, H., Koya, R.C., Ng, C., Chodon, T., Scolyer, R.A., Dahlman, K.B., et al. (2012). Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. Nat. Commun. *3*, 724.

Wan, P.T., Garnett, M.J., Roe, S.M., Lee, S., Niculescu-Duvaz, D., Good, V.M., Jones, C.M., Marshall, C.J., Springer, C.J., Barford, D., and Marais, R.; Cancer Genome Project (2004). Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell *116*, 855–867.