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Chemically Linked Vemurafenib Inhibitors Promote an Inactive BRAF^{V600E} Conformation

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Abstract

The BRAF kinase, within the mitogen activated protein kinase (MAPK) signaling pathway, harbors activating mutations in about half of melanomas and to a significant extent in many other cancers. A single valine to glutamic acid substitution at residue 600 (BRAF^{V600E}) accounts for about 90% of these activating mutations. While BRAF^{V600E}-selective small molecule inhibitors, such as vemurafenib and vemurafenib, have shown therapeutic benefit, almost all patients develop resistance. Resistance often arises through reactivation of the MAPK pathway, typically through mutation of upstream RAS, downstream MEK or splicing variants. RAF kinases signal as homo- and hetero-dimers, and another complication associated with small molecule BRAF^{V600E} inhibition is drug-induced allosteric activation of a wild-type RAF subunit (BRAF or CRAF) of the kinase dimer, a process called ‘transactivation’ or ‘paradoxical activation.’ Here we used BRAF^{V600E} and vemurafenib as a model system to develop chemically linked kinase inhibitors to lock RAF dimers in an inactive conformation that cannot undergo transactivation. This structure-based design effort resulted in the development of Vem-BisAmide-2, a compound containing two vemurafenib molecules connected by a bis amide linker. We show that Vem-BisAmide-2 has comparable inhibitory potency as vemurafenib to BRAF^{V600E} both *in vitro* and in cells, but promotes an inactive dimeric BRAF^{V600E} conformation unable to undergo transactivation. The crystal structure of a BRAF^{V600E}/Vem-BisAmide-2 complex and associated biochemical studies reveal the molecular basis for how Vem-BisAmide-2 mediates selectivity for an inactive over active dimeric BRAF^{V600E} conformation. These studies have implications for targeting BRAF^{V600E}/RAF heterodimers and other kinase dimers for therapy.

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AUTHOR CONTRIBUTIONS

M.G., and J.M. carried out biochemical and structural experiments under direction of R.M. M.A.E. and C.V. synthesized compounds under the direction of J.D.W. M.S. carried out cell-based experiments under the direction of J.V. M.G. prepared figures 1–5 and M.S. prepared figure 6. M.G., M.A.E., J.V., J.D.W. and R.M. wrote the manuscript. All authors read and approved the manuscript.

INTRODUCTION

BRAF is a notable oncoprotein within the MAPK signaling pathway, which involves phosphorylation cascades leading to protein translation and transcription factor regulation important for cell proliferation. BRAF, in contrast to the other RAF isoforms, ARAF and CRAF, has a propensity to be mutated in a significant number of cancers, and in about 50% of melanomas in particular. About 90% of BRAF mutations associated with cancer contain a single point mutation of valine to glutamic acid at residue 600 (BRAF^{V600E}), which triggers an active protein conformation in the absence of activation loop phosphorylation^{1–5}. Because of the prevalence of BRAF^{V600E} mutations in melanoma, this mutant form of the kinase has emerged as an important drug target for melanoma therapy. BRAF^{V600E}-selective inhibitors such as vemurafenib (PLX4032)^{6–7} and dabrafenib^{8–9} have been approved by the FDA based on extended overall survival in patients with metastatic BRAF^{V600E} melanoma. However, almost all patients develop drug resistance within about 6 months of treatment through diverse mechanisms, but predominantly through reactivation of the MAPK pathway, for example through mutation of upstream RAS or downstream MEK^{10–15}. To address these shortcomings, recent studies have combined BRAF and MEK inhibitors to block MAPK reactivation; this combination has been approved by the FDA for the treatment of BRAF mutant melanoma. However, resistance still develops and this strategy has limited activity in a subset of melanomas with acquired resistance to RAF or MEK inhibitor monotherapy, particularly in the context of increased MAPK signaling^{15–18}.

Another drug resistance mechanism takes advantage of the fact that RAF kinases normally signal as homo- and hetero-dimers, and drug-bound BRAF in an inactive conformation is able to allosterically shift the associated non-drug bound wild-type BRAF or CRAF subunit into an activate conformation in a process called “transactivation” or “paradoxical activation” to promote MAPK signaling²⁷. Paradox breaker inhibitors, in which the sulfonamide tail of vemurafenib has been altered to break paradoxical activation have been reported¹⁹, however resistance pathways involving dimerization with these inhibitors can still arise. RAF dimerization is promoted by RAS activation^{20, 21}, and while BRAF^{V600E} can signal as a monomer, cells that have activated RAS can display paradoxical activation upon treatment with BRAF inhibitors²¹. Indeed, transactivation through RAF dimers accounts for a common intrinsic drug resistance mechanism in melanomas^{22–29}, as upregulating RAS mutations are common within drug resistant melanomas²⁶. In addition, acquired resistance to BRAF^{V600E} can occur through the expression of a 61kDa-spliced variant of BRAF^{V600E} with enhanced dimerization and transactivation properties¹¹. Consistent with the importance of RAF dimerization, a single R509H mutation that disrupts BRAF dimerization is shown to prevent drug-induced transactivation³⁰. Together, these studies highlight the importance of RAF dimerization for BRAF^{V600E}-mediated melanoma, and suggest that novel approaches to specifically target RAF dimers may have therapeutic value.

In the study reported here, we used BRAF^{V600E} dimers in the active conformation as a model system to ask if chemically linked vemurafenib inhibitors could shift RAF dimers into an inactive conformation. We show that chemically linked vemurafenib inhibitors promote an inactive BRAF^{V600E} dimeric conformation, implicating that a similar strategy

can be employed to target BRAF^{V600E}/RAF^{WT} dimers for inhibition of transactivation in the MAPK pathway.

RESULTS

Chemically linked vemurafenib molecules specifically and potently disrupt active BRAF^{V600E} dimers

Vemurafenib was the first selective BRAF inhibitor approved to treat BRAF^{V600E} melanoma based on efficacy and improved overall survival; however, responses are transient due to the emergence of resistance in virtually all patients, demonstrating the necessity for more effective drugs/therapies, particularly those that directly address the issue of transactivation^{6,9}. Therefore, we hypothesized that two vemurafenib molecules chemically linked in an appropriate fashion could inhibit a RAF dimer and thus prevent paradoxical activation. Of the 45 BRAF kinase domain crystal structures available in the PDB, only one (4WO5)³¹ is not present in what is presumed to be a biologically relevant dimer (Figure 1a). In the BRAF dimer, residue Arg509 makes dimer-stabilizing H-bonds to the backbone carbonyl oxygens of T508 and R506 of the other BRAF subunit of the dimer, which contributes to orienting the α C-helix in a conformation that facilitates catalysis (Figure 1b)³¹. The dimer structures reveal that the kinase active sites face away from each other and are separated by about $\sim 30\text{\AA}$ (Figure 1a). Based on this observation, we reasoned that a linker of $\sim 30\text{\AA}$ could enable an inhibitor to bind both active sites of the observed dimer simultaneously, while a shorter linker might also be able to “trap” BRAF into another, potentially inactive dimer conformation. With this in mind, we prepared linked vemurafenib molecules in which the tether contained 2 ($\sim 10\text{\AA}$) to 6 ($\sim 30\text{\AA}$) polyethylene glycol (PEG) units (see supplemental methods section for details). (Figure 1c).

The potencies of the linked vemurafenib inhibitors against BRAF^{V600E} and BRAF^{WT} were evaluated *in vitro* using an ELISA assay that measures phosphorylation of the BRAF substrate GST-MEK1. We found that all but the linked compound with the fewest PEG units (Vem-2-Vem) had potencies within 2-fold of unlinked PLX4720 (an analogue of vemurafenib without a phenyl group, referred to herein as PLX) inhibitor against BRAF^{V600E}. In control studies, we also demonstrated that PLX4720 (PLX) and PLX4032 (Vemurafenib, or Vem) have potencies within about 2-fold of each other (Figure S1). Interestingly, all of the linked compounds had considerably poorer potency against BRAF^{WT} (Figures 2a and 2b, respectively). This data demonstrates that the potency of the chemically linked vemurafenib inhibitors against BRAF^{V600E} is relatively insensitive to linker length between 3 and 6 units. Unexpectedly, linkers of all lengths significantly reduced the potency of vemurafenib against BRAF^{WT}, suggesting that the linked vemurafenib inhibitors are binding to two BRAF^{V600E} subunits in a way that differed from the active dimer conformation. This hypothesis was also corroborated by the data showing that all the linker lengths, except the shortest $10\text{\AA}$ length did not significantly affect linked inhibitor potency.

As RAF dimerization is predicted to increase the thermal stability of the protein, we performed a thermal stability assay of BRAF^{V600E} with and without inhibitors as a readout for BRAF^{V600E} dimerization. We carried out these studies by measuring the fluorescence of the reporter molecule sypro orange as it binds hydrophobic residues of BRAF, which

becomes exposed as thermal denaturation occurs³³. These studies reveal that while unlinked PLX increased the melting temperature of BRAF^{V600E} by about 18 °C, the linked vemurafenib compounds produce a significant but more modest 2–9 °C increase in melting temperature (Figure 2c and Figure S2). These results are consistent with a model whereby the linked vemurafenib compounds change the configuration of the two BRAF^{V600E} molecules relative to the configuration of the active dimer conformation.

To evaluate the cellular activity of the linked vemurafenib compounds, we treated melanoma cells that harbored BRAF^{V600E} (WM983B) or BRAF^{WT} (WM3000) with unlinked or linked vemurafenib compounds. Consistent with the *in vitro* studies, Vem-6-Vem showed comparable inhibitory activity on cell viability in BRAF^{V600E}-mutant melanoma cells, but showed no significant inhibitory activity in BRAF^{WT}-melanoma cells (Figure 2d). These results also demonstrate that the linked vemurafenib compounds are able to enter cells and exhibit on-target activity.

Crystal structures of Vem-6-Vem and Vem-3-Vem demonstrate that linked vemurafenib inhibitors force BRAF^{V600E} subunits into a face-to-face inactive dimer conformation

To determine the molecular basis for BRAF^{V600E} inhibition by the linked vemurafenib compounds, we determined the crystal structure of the BRAF^{V600E} kinase domain bound to Vem-6-Vem. The crystals formed in the space group P2₁ with two BRAF^{V600E} molecules in the asymmetric unit, and the structure was determined by molecular replacement using 4WO5 as a search model and was refined to 2.3 Å resolution with good geometry and refinement statistics (Table 1). As expected, the refined BRAF^{V600E}/Vem-6-Vem structure shows electron density corresponding to a vemurafenib molecule bound in each enzyme active site, although electron density is not observed for the PEG linker, which was presumably disordered in the crystal structure (Figure 3a). Indeed, the last resolvable atom closest to the PEG linker, a carbon attached to the first oxygen in the linker portion, has a B factor of 34 Å², while the average B factor of the structure is 24 Å². As expected from the solution studies, the BRAF^{V600E}/Vem-6-Vem structure did not adopt the active RAF dimer conformation typically seen in RAF crystal structures, where the active sites are facing away from each other (Figure 1a). Instead, the BRAF^{V600E}/Vem-6-Vem dimer structure reveals that one protein subunit of the dimer is flipped about 180° relative to its position in the active RAF dimer such that the active sites are facing towards each other (Figure 3a). Figure 3b shows one subunit of the Vem-6-Vem structure superimposed with one subunit of the 1UWH biologically relevant dimer, demonstrating the different positions of the associated protein pair and αC-Helix, highlighted in orange, in both structural configurations. As a consequence of the altered configuration of the BRAF^{V600E}/Vem-6-Vem structure, key elements of the active RAF dimer are absent. Notably, R509 is surface exposed and thus does not participate in dimer interactions. Significantly, both BRAF molecules in the BRAF^{V600E}/Vem-6-Vem dimer structure are in an inactive dimer conformation as characterized by a shift of the C-helix to a more open configuration and an ordering of the activation segment into a helical conformation, referred to as AS-H1³¹ (Figure 3c). A similar inactive kinase conformation was noted in a recently reported BRAF monomer bound to vemurafenib (accession code 4WO5)³¹ (Figure 3c). Notably, while the same inactive kinase configuration is observed between BRAF^{V600E}/Vem-6-Vem and 4WO5, the contact areas

between both subunits in the asymmetric unit are different, despite being crystallized under similar conditions (details can be found in the methods section). This observation suggests that the crystallographically observed inactive kinase conformation results from a destabilization of the active BRAF dimer by Vem-6-Vem rather than by crystal contacts.

Based on previous crystallographic and solution studies demonstrating that the BRAF^{V600E} kinase domain favors a dimeric state, while BRAF^{WT} favors a monomeric state, and analogies with other kinases, it was proposed that the AS-H1 helix is a driver of the inactive monomeric BRAF^{WT} conformation³¹. Based on this model, interactions between the AS-H1 helix and the C-helix are proposed to force the C-helix into an inactive conformation. BRAF^{WT} activation loop phosphorylation or BRAF^{V600E} mutation is proposed to destabilize interactions between the AS-H1 helix and the C-helix, causing the C-helix to shift into the active and dimeric form. The BRAF^{V600E}/Vem-6-Vem structure reveals that the AS-H1 helix is formed, even with the glutamate present in the BRAF^{V600E} mutant. While the glutamate is within close contact to Ala497 (3.5 Å) (Figure 3d), the inactive conformation is still favored, and present in both BRAF molecules in the asymmetric unit. Alignment of the BRAF^{V600E}/Vem-6-Vem complex with 1UWH in Figure 3d reveals that the BRAF^{V600E} mutation is still able to facilitate formation of AS-H1 helix and the inactive kinase conformation because Glu501 shifts away from Glu600, thus avoiding potentially destabilizing interactions. These observations argue against the AS-H1 helix being a driver of BRAF^{V600E} activation and instead for the inherent ability of the linked Vem-6-Vem inhibitor to drive formation of the inactive BRAF^{V600E} kinase conformation.

To determine if the inactive kinase conformation observed in the BRAF^{V600E}/Vem-6-Vem structure was dependent on the linker length between the vemurafenib molecules and to potentially capture the path of the linker, we prepared crystals of BRAF^{V600E} bound to Vem-3-Vem. Vem-3-Vem is estimated to have a linker length of ~15 Å, compared to the estimated linker length of ~30 Å, for the Vem-6-Vem compound. Crystallization of the BRAF^{V600E}/Vem-3-Vem complex under the conditions that produced the BRAF^{V600E}/Vem-6-Vem structure was unsuccessful, and we hypothesize that the lack of a highly dynamic linker altered crystal contacts. We were able to crystallize the BRAF^{V600E}/Vem-3-Vem complex in the P2₁2₁2₁ space group containing four BRAF protomers (two inhibitor-linked dimers) in the asymmetric unit cell. The structure was determined by molecular replacement using the BRAF^{V600E}/Vem-6-Vem structure and refined to 2.19 Å with good geometry and refinement statistics (Table 1). Despite the fact that the BRAF^{V600E}/Vem-3-Vem complex was in a different crystal environment than the BRAF^{V600E}/Vem-6-Vem complex, the two BRAF^{V600E}/Vem-3-Vem complexes in the asymmetric unit show the same face-to-face and inactive kinase conformation as BRAF^{V600E}/Vem-6-Vem (RMSD of 1.98 Å² for all common atoms) (Figure 4a). In particular, the BRAF^{V600E}/Vem-3-Vem complex shows the same C-helix shift and presence of the AS-H1 segment characteristic of the inactive kinase conformation (Figure 3c). This observation further supports the conclusion that the inactive kinase conformations observed in the BRAF^{V600E} crystal structures bound to the chemically linked vemurafenib inhibitors are promoted by the linked inhibitors themselves and not crystal packing forces.

Functionalization of the linker can increase the potency of chemically linked vemurafenib over unlinked PLX

Unlike the ~30 Å, linker of the Vem-6-Vem compound in the BRAF^{V600E}/Vem-6-Vem structure, which was disordered, the shorter ~15 Å, linker of the Vem-3-Vem compound could be reliably traced into the electron density map (Figure 4b). Interestingly, the linker region is in proximity to residues Gln461 and His539 of both BRAF^{V600E} subunits of the dimer (Figure 4c). We reasoned that functionalization of the linker region to introduce hydrogen bond acceptors and/or donors could introduce stabilizing protein-linker interactions to increase the potency of the linked vemurafenib inhibitors for BRAF^{V600E}. With this in mind, we designed a set of second-generation inhibitors, in which the linker region was functionalized. We reasoned that adding carbonyl and amine functional groups within the linker would add the hydrogen bond acceptors and/or donors with which Gln461 and His539 could interact, potentially strengthening the interaction of the inhibitor and, in turn, further stabilizing the forced inactive BRAF^{V600E} conformation. We prepared three new diglycolic diamide linked-dimers, in which the number of methylenes between the diglycolic amide nitrogens and the phenyl ring of the vemurafenib core was varied (details on synthesis can be found in the Supplementary Information). The three analogs synthesized consisted of an N-aryl diamide, as well as N-benzylic, and N-homobenzylic diamide linkers (Figure 5a). These compounds allowed us to examine the effect of linker length, in addition to increasing electronic density of the carbonyl functions due to amide resonance. We reasoned that this effect would facilitate increased interaction of the dimers with Gln461 and His539, relative to the analogous ketone linkers.

We assayed the inhibitor activity of the three bis amide-linked vemurafenib compounds against BRAF^{V600E} and BRAF^{WT} *in vitro* using the ELISA assay used for first generation inhibitors and found that all three amide-linked vemurafenib compounds lacked potency against BRAF^{WT}, as expected (Figure S3). However the ability of the compounds to inhibit BRAF^{V600E} varied significantly (Figure 5b). Vem-BisAmide-1 inhibited BRAF^{V600E} poorly compared to PLX, with an IC₅₀ of ~3.62 μM compared to 115 nM for PLX measured under identical conditions, and Vem-BisAmide-3 demonstrated an IC₅₀ value of 195 nM, mimicking Vem-3-Vem and Vem-6-Vem. Vem-BisAmide-2 showed the greatest potency of the bis amide-linked vemurafenib compounds for BRAF^{V600E}, with an IC₅₀ of 33.5 nM, about 3–5 fold higher than PLX and the PEG-linked vemurafenib inhibitors, but comparable to vemurafenib alone (Figure S1). We hypothesized that the geometry of Vem-BisAmide-2 is most optimal for inhibiting the inactive BRAF^{V600E} dimer conformation, through more restricted movement of the linker and interactions with residues Gln461 and/or His539 of BRAF^{V600E}. Consistent with the findings of the activity assay, a thermal stability assay demonstrates that Vem-BisAmide-2 increases the thermal stability of BRAF^{V600E} more than Vem-6-Vem and on the same level as unlinked PLX (Figure 5c). These results demonstrate that judicious functionalization of the linker can significantly increase BRAF^{V600E} inhibitor potency.

Crystal structure of BRAF^{V600E}/Vem-BisAmide-2 complex reveals the molecular basis for inhibition of BRAF^{V600E} dimers

We were able to successfully crystallize Vem-BisAmide-2 bound to BRAF^{V600E}. The crystals were isomorphous to the BRAF^{V600E}/Vem-3-Vem structure and thus contained four BRAF protomers (two inhibitor-linked dimers) in the asymmetric unit. The structure was determined by molecular replacement using the BRAF^{V600E} monomer from the BRAF^{V600E}/Vem-3-Vem structure and refined to 2.70 Å resolution with good geometry and refinement statistics (Table 1). As predicted, each enzyme active site was occupied by a vemurafenib molecule and the C-helix and the AS-H1 helix mimicked the conformation shown in the other structures with inactive conformations. The Vem-BisAmide-2 linker density was more pronounced than that of Vem-3-Vem despite the more limited resolution, consistent with a more rigid linker. The linker region of Vem-BisAmide-2 shows that the amide carbonyls are making interactions with Gln461 and His539 residues. While the His539 residues make van der Waals contact to methyl groups in the linker, Gln461 residues hydrogen bond to the amide carbonyl groups of the linkers, with the nitrogen of the amide groups of the glutamine within close proximity of the carbonyl groups of the inhibitor's amide linker (Figure 6a). In the two BRAF^{V600E} dimers in the asymmetric unit, the electron density for Gln461 is weak or unclear for one of the two dimer subunits (Figure S4). This observation suggests that stabilization of one Gln461-linker interaction does not synergistically nucleate the second Gln461-linker interaction and thus the interactions are independent. Nonetheless, the biochemical data argues for the importance of the Gln461-linker interaction in increasing inhibitor activity of the linked vemurafenib molecules, and we therefore hypothesize that only one Gln461/linker interaction contributes to formation of the protein/inhibitor complex.

To evaluate the contribution of the second vemurafenib molecule of the Vem BisAmide-2 compound on inhibitor potency and promotion of the inactive dimeric BRAF^{V600E} conformation, a control inhibitor (Vem-BisAmide-4, Figure 5a) containing the BisAmide-2 linker but only one Vemurafenib molecule was synthesized and tested against BRAF^{V600E} and BRAF^{WT} in activity and thermal stability assays. These data revealed comparable potency to PLX and Vem-BisAmide-2 (Figure 5B and Figure S5), but a thermal melting temperature closer to PLX than Vem-BisAmide-2 (Figure 5C Figure S6). These data suggest that PLX and Vem-BisAmide-4 are promoting comparable BRAF^{V600E} configurations that are distinct from the BRAF^{V600E} complex with Vem-BisAmide-2, and consistent with the conclusion that the second vemurafenib molecule in the bivalent Vem-BisAmide-2 inhibitor plays a key role in promotion of the inactive dimeric BRAF^{V600E} conformation.

Mutagenesis studies were performed to probe the importance of Gln461 for BRAF^{V600E} inhibition by Vem-BisAmide-2. We prepared the BRAF^{V600E/Q461A} mutant and assessed its ability to be inhibited by Vem-BisAmide-2 relative to BRAF^{V600E/Q461A}. IC₅₀ curves of Vem-BisAmide-2 against BRAF^{V600E} and BRAF^{V600E/Q461A} demonstrated that the Gln461A mutation shifts the IC₅₀ from ~56 nM to ~464 nM, respectively, an almost 10 fold difference (Figure 6b). In contrast PLX shows a comparable IC₅₀ of ~80–130 nM for both BRAF^{V600E} and BRAF^{V600E/Q461A} (Figure 6b). These studies highlight the importance of the role of BRAF Gln461 for inhibition by Vem-BisAmide-2.

Vem-BisAmide-2 can promote the formation of BRAF^{V600E} dimers in solution

In order to validate that Vem-BisAmide-2 can mediate an inactive BRAF^{V600E} dimer in solution as was observed in the crystal structures, we performed analytical ultracentrifugation experiments with and without Vem-BisAmide-2. For these experiments, we utilized an R509H point mutation, previously shown to disrupt the active BRAF^{V600E} dimer³⁰. Control sedimentation velocity experiments demonstrated that while 10 μ M BRAF^{V600E} sediments as a dimer, BRAF^{V600E/R509H} sediments as a monomer. However, the addition of 5 μ M of Vem-BisAmide-2 to BRAF^{V600E/R509H} shifts the sedimentation coefficient of BRAF^{V600E/R509H} to two distinct populations, matching both a monomer and a dimer (Figure 6c). Vem-BisAmide-2 concentrations of 10 μ M and 15 μ M were also tested, and increasing concentrations of inhibitor were correlated with more dimer formation for BRAF^{V600E/R509H} while BRAF^{V600E} consistently sediments as a dimer, regardless of whether Vem-BisAmide-2 is added or not. To further quantify the sedimentation velocity experiments, sedimentation equilibrium experiments were performed. Log plots of the data are shown in Figure 6d, where the slope of the line is proportional to the estimated molecular weight of the species in solution. BRAF^{V600E/R509H} with no inhibitor bound gives a single species with an estimated molecular weight of 40kD, where the predicted molecular weight of the monomer is ~35kD, indicative of predominantly monomer formation. This species also has a slope that corresponds to that of the predicted line of an ideal monomer. However, when Vem-BisAmide-2 is added, the single species molecular weight is ~70kD, precisely the predicted molecular weight of a BRAF dimer. When BRAF^{V600E/R509H} is incubated with PLX, the ideal fit molecular weight is 40kD, demonstrating that the presence of the unlinked inhibitor is not able to alter the monomeric state of the kinase. These studies validate the promotion of BRAF^{V600E} dimer formation by Vem-BisAmide-2, consistent with the crystallographic data demonstrating that it promotes the formation of BRAF^{V600E} dimers in the inactive conformation.

We also performed sedimentation velocity experiments with BRAF^{V600E/R509H} and Vem-BisAmide-4, which contains a single vemurafenib moiety, and demonstrated that the BRAF^{V600E/R509H}/Vem-BisAmide-4 complex adopts a monomeric configuration (Figure S7) supporting our conclusion that the second vemurafenib molecule in the bivalent Vem-BisAmide-2 inhibitor plays a key role in promotion of the inactive dimeric BRAF^{V600E} conformation.

Vem-BisAmide-2 is able to enter melanoma cells to selectively target BRAF^{V600E} over BRAF^{WT}

To determine if Vem-BisAmide-2 can selectively inhibit BRAF^{V600E} in cells, we treated BRAF^{V600E} (WM983B) or BRAF^{WT} (WM3000) melanoma cells with Vem-BisAmide-2, vemurafenib and other selected linked vemurafenib inhibitors. We observed that each of the inhibitors tested showed comparable dose-response inhibition of cell growth (Figures 7a and 7b, Figures S8) in cells harboring mutant BRAF^{V600E} (WM983B) but not in cells harboring BRAF^{WT} (WM3000). Furthermore, we found that the linked compounds blunted MAPK signaling (assessed by pERK and pMEK levels) (Figure 7C, top), but less potently than the unlinked PLX or the MEK inhibitor PD901. Of note, none of the RAF inhibitors tested blocked MAPK signaling in BRAF^{WT} cells (Figure 7C, bottom). These studies demonstrate

that Vem-BisAmide-2 can enter cells to selectively inhibit BRAF^{V600E}. The fact that BRAF^{V600E} homodimer species are unlikely to exist in BRAF^{V600E}-mutant melanoma cells⁴⁸, is consistent with the observation that unlinked and linked-vemurafenib inhibitors show comparable BRAF^{V600E} inhibitory potency in cells.

DISCUSSION

In summary, we demonstrate that appropriately covalently linking two vemurafenib molecules produces an inhibitor with enhanced potency and selectivity for targeting BRAF^{V600E} over BRAF^{WT}, and most significantly, a shift from an active to inactive BRAF^{V600E} conformation, which is trapped in a dimeric state that cannot undergo paradoxical activation. To our knowledge, this is the first inhibitor shown to promote such an inactive BRAF^{V600E} dimeric conformation. While other sulfonamide inhibitors have been shown to favor an inactive monomeric state of BRAF^{WT}, the same is not true for BRAF^{V600E}, where sulfonamide inhibitors such as vemurafenib promote an active dimeric conformation³¹. In addition, recently reported “paradox breaker” inhibitors, such as PLX7904, PLX7922 and PLX5568, were shown to disfavor dimerization. However, crystal structures of these inhibitors bound to BRAF^{V600E} (4XV1 and 4XV3) and BRAF^{WT} (4XV9) still demonstrate packing in a biologically relevant dimer in the active conformation, characterized by the active conformation of the C-helix and the absence of the AS-H1 helix¹⁹. The two BRAF^{V600E} co-crystal structures also have only one inhibitor molecule bound per dimer pair, with the second protomer binding site inhibitor-free and in the active conformation, suggesting that these inhibited dimers may be susceptible to paradoxical activation.

The studies reported here have important implications for developing analogous linked inhibitors to target BRAF^{V600E}/RAF^{WT} heterodimers through the substitution of a pan RAF inhibitor for one of the vemurafenib molecules and appropriate alterations of the linker properties. Such inhibitors would be useful as molecular probes to study the biological importance of RAF dimerization in MAPK signaling and have the potential as novel molecules to target BRAF^{V600E} resistant melanomas. Targeting heterodimers with a linked inhibitor rather than combinations of inhibitors has the potential to demonstrate more selectivity, leading to lower doses and potentially a more prolonged response.

The linked kinase inhibitor strategy described herein also has the potential to be extended to other kinase systems that utilize dimeric forms for signaling and allosteric regulation, such as RTKs, or two different interacting kinases such as RAF and MEK. This strategy could enhance inhibitor synergy or dosing issues that usually come with combination therapies^{15–18}. While these dimeric structures are larger than 500 Da (1067 Da for Vem-BisAmide-2) and therefore do not follow the Lipinski rules⁴⁰, our studies demonstrate that such molecules have good uptake into cells, can engage their substrates and show potent cellular activity. In another example of successfully employing linked inhibitors, Illendula *et. al.* recently reported on PEG-linked inhibitor of the dimeric transcription factor CBF β -SMMHC fusion protein to delay leukemia in mice⁴¹.

Taken together, the studies reported here demonstrate that chemically linked RAF inhibitors hold promise for eliciting a prolonged response to BRAF^{V600E}-driven melanoma, either administered alone or in combination with immunotherapy⁴², and have implications for targeting other kinase dimers for therapy.

METHODS

Plasmids

DNA encoding the BRAF kinase domain residues 448–723 containing 16 solubilizing mutations⁷ (I543A, I544S, I551K, Q562R, L588N, K630S, F667E, Y673S, A688R, L706S, Q709R, S713E, L716E, S720E, P722S, and K723G) permitting kinase domain overexpression in bacteria was ordered from Epoch Biolabs and cloned into a Pet28a(+) vector encoding an N-terminal His tag and a thrombin cleavage site between the protein and His tag. This construct was used as a template to create His-tagged BRAF^{V600E-16M}, BRAF^{R509H-16M} and BRAF^{V600E/R509H-16M} mutants. These proteins were used in Analytical Ultracentrifugation Sedimentation Velocity and Sedimentation Equilibrium experiments, and Thermal Stability assays. DNA encoding the BRAF^{V600E-16M} construct was subcloned into a PRSF vector containing a TEV protease-cleavable GST-tag and used for crystallization.

DNA encoding the BRAF kinase domain residues 442–724 was used as a template to prepare BRAF^{WT}, BRAF^{V600E} and BRAF^{V600E/Q461A} were cloned into a Pfastbac dual vector with mouse p50^{cdc37} full length as an expression chaperone for protein expression in baculovirus infected Sf9 insect cells and used for *in vitro* kinase assays. Full length human MEK1 with an N-terminal GST fusion tag and a C-terminal His tag in a pGex-3t vector was provided by Dr. Michael Olson (Beatson Institute for Cancer Research, Glasgow, UK) and was used for our *in vitro* kinase assays.

Protein Purification

His-tagged BRAF^{-16M} proteins were expressed in Rosetta2 BL21 bacterial expression cells at 37 °C and induced with 1mM IPTG overnight at 18°C, spun down the next day and lysed in Lysis Buffer (50 mM Potassium Phosphate, pH 7.0, 250 mM NaCl, 5 mM Imidazole) and 1 mM PMSF. The lysate was then spun down at 19000 r.p.m. and the supernatant was added to 5 mL TALON metal affinity resin (Takara) and left to incubate at 4°C for 1 hour. The supernatant was then eluted, the column washed with 1L of Lysis Buffer and the BRAF^{-16M} proteins eluted with Lysis Buffer supplemented with 250mM Imidazole. Protein were then dialyzed into Lysis Buffer without imidazole but with 5 mM EDTA and NaCl adjusted to 5 mM NaCl, prior to application on a 5 mL SP Sepharose anion exchange column, followed by washing in the same buffer and elution in lysis buffer with 0 mM NaCl to 1 M NaCl gradient. Peak fractions were pooled, concentrated, and applied to a Superdex S200 gel filtration column in a final buffer of 20 mM HEPES pH 7.0, 150 mM NaCl, 10 mM Dithiothreitol and 5% glycerol. Protein was flash frozen in liquid nitrogen and stored for future use.

GST-tagged BRAF^{V600E-16M} protein was expressed in bacteria as described for the His-tagged proteins, lysed Lysis Buffer 2 (50 mM KPi pH 7.0 and 250 mM NaCl) and incubated on Glutathione Resin at 4 °C for 1 hr. The protein on resin was then washed and left on the resin and cleaved with TEV protease overnight and eluted the next morning in Lysis Buffer 2 with 25 mM NaCl, run over both SP Sepharose and Q Sepharose ion exchange columns in tandem and the flow through collected. The protein was then concentrated using a 10kDalton cutoff centrifugal filter unit (Millipore) and chromatographed on a Superdex S200 gel filtration column in a final buffer of 20 mM HEPES pH 7.0, 150 mM NaCl, 10 mM Dithiothreitol and 5% glycerol. Protein eluted as a mix of dimer and monomer and both species were pooled together, concentrated to 10mg/mL (~320 μM) and used immediately for crystallization.

BRAF^{WT}, BRAF^{V600E}, and BRAF^{V600E/Q461A} were overproduced as N-terminally His-tagged proteins in insect cells essentially as previously described³². Briefly, protein constructs were coexpressed with p50^{cdc37}, pelleted, suspended in Lysis Buffer 3 (25 mM Tris 8.0, 250 mM NaCl, 5 mM Imidazole, and 10% glycerol) treated with Complete EDTA-free protease inhibitor cocktail tablets (Roche) and DNaseI, lysed, centrifuged at 19000 r.p.m and added to TALON metal affinity resin and incubated for 1 hr at 4 °C. The protein on the resin was then washed extensively with Lysis Buffer 3 and then eluted with 25 mM Tris pH 7.5, 250 mM NaCl, 250 mM Imidazole, and 10% Glycerol. The protein was then diluted in low salt buffer containing 1 mM EDTA and 1 mM Dithiothreitol and run on an SP Sepharose column, eluted with a salt gradient from 0 mM NaCl to 1 M NaCl. Peak fractions were pooled and run on a Superdex S200 gel filtration column and stored in a final buffer of 25 mM Tris 8.0, 300 mM NaCl, 1 mM Dithiothreitol and 10% glycerol. Protein was concentrated to ~0.5mg/mL and flash frozen in liquid nitrogen and stored for later use for ELISA kinase assays.

GST-MEK1 fusion protein used as substrate in ELISA assays was prepared essentially as previously described³². Briefly, protein was expressed in BL21 (Gold) cells at 37 °C and induced with 0.5 mM IPTG at 15 °C overnight. The cells were then harvested and resuspended in Lysis Buffer 4 (20 mM Hepes pH 7.5, 500 mM NaCl, 10 mM BME, 10 mM Imidazole, 5% Glycerol) supplemented with 1 mM PMSF and DNaseI. The lysate was sonicated and spun down, and the supernatant was added to Ni-NTA resin and incubated for 1 hr at 4 °C. The resin was then washed extensively with Lysis Buffer 4 and eluted with Lysis Buffer 4 supplemented with 250mM Imidazole. Peak fractions were then concentrated as described above and loaded onto a Superdex S200 gel filtration column in a final buffer of 20 mM HEPES pH 7.0, 150 mM NaCl, 10 mM BME, and 5% glycerol. The protein eluted off the sizing column in two separate populations, and the second peak corresponding to a GST-MEK dimer was collected, concentrated to ~20mg/mL, and flash frozen in liquid nitrogen and stored for later use.

Crystallization, Data Collection, and Structural Analysis

BRAF^{V600E-16M} at 10 mg/mL was mixed with a 10 mM (in 100% DMSO) stock solution of inhibitor (Vem-6-Vem, Vem-3-Vem, and Vem-BisAmide-2, respectively) to a final inhibitor concentration of 500 μM and trays were set up screening around a crystallization condition

of 100 mM Tris pH 8.5, 14% PEG Monomethyl Ether 2000, and 200 mM Trimethyl Amine N-oxide Dihydrate for Vem-6-Vem and a condition of 100 mM Tris 8.5, 5% ethanol, and 2% Benzamidine HCl for Vem-3-Vem and Vem-BisAmide-2 using the hanging-drop vapor diffusion method at 4°C. Crystals were flash frozen in cryo-protected mother liquor containing 20% glycerol. X-ray diffraction data were collected at a wavelength of 0.98 Å at the Advanced Photon Source (beamline 24-ID-E) for BRAF^{V600E-16M} inhibitor crystals with Vem-6-Vem and Vem-3-Vem, and with Vem-BisAmide-2 was collected in house using the Rigaku MicroMax-007HF with a mar CCD 165mm detector. The BRAF^{V600E-16M} inhibitor crystal with Vem-6-Vem was processed using HKL-2000³⁴, Vem-3-Vem was processed using XDS³⁵ and Vem-BisAmide-2 was processed using HKL-2000³⁴.

All three co-crystal structures were determined by molecular replacement in PHENIX using phaser^{36, 37}. The BRAF^{V600E-16M}/Vem-6-Vem structure was determined using PDB 4WO5³¹ as a search model in Phaser and the BRAF^{V600E-16M}/Vem-3-Vem and BRAF^{V600E-16M}/Vem-BisAmide-2 structures were determined using the BRAF^{V600E-16M}/Vem-6-Vem structure as a search model. Molecular Replacement search models had the inhibitor removed from them, and for the structure determination of BRAF^{V600E-16M}/Vem-6-Vem, the C-helix was removed from the search model. For the structure determinations of BRAF^{V600E-16M} with Vem-3-Vem and Vem-BisAmide-2, the C-helix was included in the molecular replacement model but deleted during refinement and rebuilt manually after PHENIX refinement. Model building and refinement were performed using Coot and PHENIX^{38, 39}. For all three structures, NCS was used, as multiple BRAF monomers were present in the asymmetric unit. Simulated Annealing omit maps were generated by taking the fully built model, deleting the inhibitor, and running through PHENIX refine using simulated annealing in the refinement. The coordinates for the Vem-6-Vem inhibitor were generated by downloading the PDB file for Vemurafenib, editing it using the REEL program in PHENIX to edit the molecule, and then running through ELBOW⁴³. The coordinates for the inhibitors Vem-3-Vem and Vem-BisAmide-2 were generated using ChemDraw, saving as a pdb file and running through ELBOW to generate a cif file. Table 1 statistics were generated using PHENIX validation tools⁴⁴.

The two sets of “dimers” in the asymmetric unit of the BRAF^{V600E-16M}/Vem-3-Vem and BRAF^{V600E-16M}/Vem-BisAmide-2 structures are also making interactions with one another, with a Benzamidine molecule buried within the protein-protein interface. Benzamidine Hydrochloride was a crystal additive, and we reason that it facilitated crystallization by stabilizing crystal contacts in this crystal form.

In Vitro Kinase Assay

Compound inhibition of BRAF^{WT} and BRAF^{V600E} were assessed using an ELISA assay performed essentially as previously described³². Briefly, GST-MEK fusion protein was diluted 3:1000 in Tris-Buffered Saline treated with 0.05% Tween-20 (TBST) and diluted MEK was added to a glutathione-coated 96 well plate (Pierce #15240) and incubated at room temperature for 1 hour with shaking. BRAF was diluted from 0.5 mg/mL frozen stocks 1:1000 in 50 mM Hepes pH 7.0 and 50 mM NaCl and treated with inhibitor at various concentrations and incubated with GST-MEK. Glutathione plates were washed extensively

and the protein-inhibitor mixture was then added to the plate with 100 μ M final concentration of ATP in a buffer containing 50mM HEPES pH 7.0, 200 mM NaCl, and 10 mM $MgCl_2$. The plate was then incubated at 37°C for 30 minutes, the reaction was then discarded and the plate was washed with TBST extensively and a 1:8000 dilution of primary antibody (p-MEK1/2 (S217/S221) Rabbit Antibody (Cell signaling)) was added to the plate and incubated for 1 hour. The plate was then treated with stringent TBST washes and then incubated with a 1:10000 dilution of secondary antibody (Goat Anti-Rabbit IgG (H+L)-HRP (BioRad)) for 1 hour. The plate was then washed and Supersignal ELISA Pico Chemiluminescent Substrate (Pierce #37069) was added and the plate read on a Perkin Elmer EnVision. Each curve was repeated in duplicate or triplicate, normalized using GraphPad Prism by selecting the largest value as the maximum and the lowest value as the minimum, and used to calculate IC_{50} values by using a log (inhibitor) vs. response fit on Prism 5.0 (GraphPad). IC_{50} values of PLX monomer, first generation Vem-linked inhibitors, and Vem-BisAmide-2 against BRAF^{V600E} were calculated using data from two separate experiments, each performed in duplicate. Error bars are indicative of the SEM of each point, and 95% confidence intervals of the IC_{50} values are listed in the figure legend, as calculated using GraphPad Prism.

Thermal Stability Assays

Frozen aliquots of BRAF^{V600E-16M} proteins were thawed and diluted in Thermal Stability Buffer (25 mM Hepes pH 7.0, 150 mM NaCl) to a final concentration of 5 μ M (0.2 mg/mL) and 15 μ L were added to the selected wells of a MicroAmp Optical 384 well plate (Applied Biosystems). Sypro Orange (5000X stock, ThermoFisher Scientific) was diluted 1:300 and 4 μ L of that diluted stock was added to each well. 1 μ L of inhibitor in 100% DMSO was added to each well to a final concentration of 50 μ M and the plate was spun down and heated from 20 °C to 95 °C using a qPCR (ABI 7900 RealTime PCR) with a 2% ramp rate. Fluorescent readings were recorded every 2 minutes. Melting curves were generated from this data and analyzed by taking the first derivative of the curve. Data were analyzed and plotted using GraphPad, with error bars indicating as SEM of each sample and 95% confidence intervals of the T_m values listed in the figure legend, both metrics were calculated using GraphPad Prism with an n=6.

Analytical Ultracentrifugation (AUC)

Sedimentation Velocity AUC was performed with a Beckman Optima XL-I at 42,000 r.p.m. Data were obtained over ~8 hours of centrifugation at 20 °C by monitoring absorbance. Concentrations of BRAF^{V600E/R509H-16M} and BRAF^{V600E-16M} were at 10 μ M while inhibitor concentration ranged from 5 μ M to 15 μ M in AUC buffer (25 mM Tris pH 7.5, 150 mM NaCl). Data was analyzed using SEDFIT to calculate a continuous c(s) distribution and data was graphed using GraphPad.

Sedimentation Equilibrium AUC was performed with the same Beckman Optima XL-I at three speeds (12,000 r.p.m, 18000 r.p.m., and 26000 r.p.m.) at three different concentrations (15 μ M, 10 μ M, and 5 μ M) of BRAF^{V600E/R509H-16M} supplemented with a 1:1 molar ratio of inhibitor at either concentration. AUC buffer from sedimentation velocity experiments was used for equilibrium experiments. Data was analyzed using Heteroanalysis to calculate an

Ideal fit molecular weight of the species in each cell and log plots of the data were subsequently graphed using GraphPad Prism. Ideal monomer and dimer fits were calculated using Heteroanalysis (Figure S8).

Cell Viability Assays

Human melanoma cell lines WM983B and WM3000 were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Corning/Cellgro) supplemented with 5% fetal bovine serum (Fisher Scientific). Cells were treated with small molecule inhibitors for 72 h and the levels of growth inhibition were examined using the MTT assay as previously described⁴⁵. Briefly, 2.5×10^3 cells/well were seeded onto a 96-well plates and grown overnight. Cells were treated with increasing concentrations of each compound (n=7) and incubated at 37C in a 5% CO₂ incubator; after 72 h of treatment, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well for 3 hours (Sigma, St. Louis, MO) followed by rapidly removing the media and solubilizing the MTT crystals using DMSO. After 10 min. the resulting absorbance was read in a plate reader (Biotek ELx800) at 490 nm.

Small molecule inhibitors

Inhibitors were purchased from Selleck Chem: BRAF inhibitor PLX4720 (cat# S1152), and MEK inhibitor PD0325901 (cat#S1036). BRAF inhibitor PLX4032 was purchased from Santa Cruz Biotechnology (cat# sc-364643)

EC₅₀ calculation—The EC₅₀ (the concentration of a drug that gives half-maximal response) values for BisAmide inhibitors are calculated from relative viability data from three separate experiments with seven replicates each. The EC₅₀ values for Vem-x-Vem dimers are calculated from relative viability data from one experiment with seven replicates each.

Western Blotting

Protein lysates were prepared and analyzed on 10% SDS-PAGE as previously described⁴⁵. In brief, whole cell protein extracts were made in TNE buffer (50 mM Tris-HCl pH 8, 250 mM NaCl, 2 mM EDTA, 1% NP-40, 1mM Na Vanadate, 1X Protease inhibitors (Complete, cat# 11697498001, Roche). 30 μ g of protein extracts were separated on 10% sodium dodecyl sulfate-polyacrylamide gels after heat denaturing the samples at 95°C for 5 min. in the presence of 1X NuPAGE sample buffer (cat# NP0007, Life Technologies). Proteins were transferred onto nitrocellulose membrane (cat# 170-4152, Biorad) using a Trans-blot Turbo transfer system (Bio-Rad). Nitro cellulose membranes were blocked for 1 hour at RT in Odyssey blocking buffer (part# 927-50000, Licor, Lincoln, NE). Incubations with primary antibodies, phospho-Erk1/2 and phospho-MEK1/2 (cat#9101 and cat# 9121, Cell Signaling Technology) and β -Actin (Cat# A5441, Sigma-Aldrich) were performed overnight at 4°C in 50% diluted blocking buffer with Tris-buffered saline (TBS; 100 mmol/L Tris-HCl, pH 7.5, and 150 mmol/L NaCl). Membranes were washed 3 times (5 min. each time) with TBS containing 1% Tween-20 (TBST) and followed by 1 hr. incubation at RT with Alexa Fluor-labeled secondary antibodies (IRDye 680LT goat-anti mouse, IRDye 800CW goat-anti

rabbit, LI-COR, Lincoln, NE) diluted (1:15000) in TBST. Immunocomplexes were visualized using Li-cor infrared Odyssey imaging system (LI-COR Lincoln, NE).

Quantification of bands on Western blots: Quantification of bands were done using Odyssey software (Licor). A boundary area was selected by drawing a rectangle around the band and the same area was chosen for all the bands in each gel. The median background correction method was selected. A quantification number is displayed under each band.

Vem-X-Vem Dimer Synthesis, BisAmide Synthesis, General Synthesis, and Inhibitor Spectral Data can be found in the Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Wellbrock C, Karasarides M, Marais R. The RAF Proteins Take Centre Stage. *Nat. Rev. Mol. Cell Biol.* 2004; 5:875–885. [PubMed: 15520807]
2. Ikenoue T, Hikiba Y, Kanai F, Tanaka Y, Imamura J, Imamura T, Ohta M, Ijichi H, Tateishi K, Kawakami T, Aragaki J, Matsumura M, Kawabe T, Omata M. Functional Analysis of Mutations Within the Kinase Activation Segment of B-RAF in Human Colorectal Tumors. *Cancer Res.* 2003; 63:8132–8137. [PubMed: 14678966]
3. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in Human Cancer. *Nature.* 2002; 417:949–954. [PubMed: 12068308]
4. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, O'Dwyer PJ, Lee RJ, Grippo JF, Nolop K, Chapman PB. Inhibition of Mutated, Activated BRAF in Metastatic Melanoma. *N Engl. J. Med.* 2010; 363(9):809–819. [PubMed: 20818844]
5. Wan PTC, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, Jones CM, Marshall CJ, Springer CJ, Barford D, Marais R. Cancer Genome Project. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell.* 2004; 116(6):855–867. [PubMed: 15035987]
6. Bollag G, Tsai J, Zhang J, Zhang C, Ibrahim P, Nolop K, Hirth P. Vemurafenib: the first drug approved for BRAF-mutant cancer. *Nat. Rev. Drug Discov.* 2012; 11(11):873–886. [PubMed: 23060265]

7. Tsai J, Lee JT, Wang W, Zhang J, Cho H, Mamo S, Bremer R, Gillette S, Kong J, Haass NK, Sproesser K, Li L, Smalley KS, Fong D, Zhu YL, Marimuthu A, Nguyen H, Lam B, Liu J, Cheung I, Rice J, Suzuki Y, Luu C, Settachatgul C, Shellooe R, Cantwell J, Kim SH, Schessinger J, Zhang KY, West BL, Powell B, Habets G, Zhang C, Ibrahim PN, Hirth P, Artis DR, Herlyn M, Bollag G. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *PNAS*. 2008; 105(8):3041–3046. [PubMed: 18287029]
8. Rheault TR, Stellwagen JC, Adjabeng GM, Hornberger KR, Petrov KG, Waterson AG, Dickerson SH, Mook RA, Laquerre SG, King AJ, Rossanese OW, Arnone MR, Smitheman KN, Kane-Carson LS, Han C, Moorthy GS, Moss KG, Uehling DE. Discovery of Dabrafenib: A Selective Inhibitor of Raf Kinases with Antitumor Activity Against B-Raf Driven Tumors. *ACS Med. Chem. Lett.* 2013; 4:358–362. [PubMed: 24900673]
9. King AJ, Arnone MR, Bleam MR, Moss KG, Yang J, Fedorowicz KE, Smitherman KN, Erhardt JA, Hughes-Earle A, Kane-Carson LS, Sinnamon RH, Qi H, Rheault TR, Uehling DE, Laquerre SG. Dabrafenib; Preclinical Characterization, Increased Efficacy when Combined with Trametinib, while BRAF/MEK Tool Combination Reduced Skin Lesions. *PLOS One*. 2013; 8(7):1–10.
10. Poulidakos P, Rosen N. Mutant Braf Melanomas-Dependence and Resistance. *Cancer Cell*. 2011; 19:11–15. [PubMed: 21251612]
11. Poulidakos PI, Persaud Y, Janakiraman M, Kong X, Ng C, Moriceau G, Shi H, Atefi M, Titz B, Gabay MT, Salton M, Dahlman KB, Tadi M, Wargo JA, Flaherty KT, Kelley MC, Misteli T, Chapman PB, Sosman JA, Graeber TG, Ribas A, Lo RS, Rosen N, Solit DB. RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). *Nature*. 2011; 480(7377):387–390. [PubMed: 22113612]
12. Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, Ludlam MJ, Stokoe D, Gloor SL, Vigers G, Morales T, Aliagas I, Liu B, Sideris S, Hoefflich KP, Jaiswal BS, Seshagiri S, Koeppen H, Belvin M, Friedman LS, Malek S. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature*. 2010; 464(7287):431–435. [PubMed: 20130576]
13. Anforth RM, Blumetti TCMP, Kefford RF, Sharma R, Scolyer RA, Kossard S, Long GV, Fernandez-Penas P. Cutaneous Manifestations of Dabrafenib (GSK2118436): A Selective Inhibitor of Mutant BRAF in Patients with Metastatic Melanoma. *Brit J. Dermatol.* 2012; 167:1153–1160. [PubMed: 22804352]
14. Haluska F, Pemberton T, Ibrahim N, Kalinsky K. The RTK/RAS/BRAF/PI3K Pathways in Melanoma: Biology, Small Molecule Inhibitors, and Potential Applications. *Semin. Oncol.* 2007; 34:546–554. [PubMed: 18083378]
15. Villanueva J, Infante JR, Krepler C, Reyes-Urbe P, Samanta M, Chen HY, Li B, Swoboda RK, Wilson M, Vultur A, Fukunaba-Kalabis M, Wubbenhorst B, Chen TY, Liu Q, Sproesser K, Demarini DJ, Gilmer TM, Martin AM, Marmorstein R, Schultz DC, Speicher DW, Karakousis GC, Xu W, Amaravadi RK, Xu X, Schuchter LM, Herlyn M, Nathanson KL. Concurrent MEK2 Mutation and BRAF Amplification Confer Resistance to BRAF And MEK Inhibitors in Melanoma. *Cell Rep.* 2013; 4(6):1090–1099. 16. [PubMed: 24055054]
16. Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, Hamid O, Schuchter L, Cebon J, Ibrahim N, Kudchadkar R, Burris HA, Falchook G, Algazi A, Lewis K, Long GV, Puzanov I, Lebowitz P, Singh A, Little S, Sun P, Allred A, Ouellet D, Kim KB, Patel K, Weber J. Combined BRAF and MEK Inhibition in Melanoma with BRAF V600 Mutations. *N Engl. J. Med.* 2012; 367(18):1694–1703. [PubMed: 23020132]
17. Greger JG, Eastman SD, Zhang V, Bleam MR, Hughes AM, Smitherman KN, Dicherson SH, Laquerre SG, Liu L, Gilmer TM. Combinations of BRAF, MEK, and PI3K/mTOR Inhibitors Overcome Acquired Resistance to the BRAF Inhibitor GSK2118436 Dabrafenib, Mediated by NRAS or MEK Mutations. *Mol. Cancer Ther.* 2012; 11:909–920. [PubMed: 22389471]
18. Johnson DB, Flaherty KT, Weber JS, Infante JR, Kim KB, Kefford RF, Hamid O, Schuchter L, Cebon J, Sharfman WH, McWilliams RR, Sznol M, Lawrence DP, Gibney GT, Burris HA, Falchook GS, Algazi A, Lewis K, Long GV, Patel K, Ibrahim N, Sun P, Little S, Cunningham E, Sosman JA, Daud A, Gonzalez R. Combined BRAF (Dabrafenib) and MEK Inhibition (Trametinib) in Patients With *BRAF*^{V600}-Mutant Melanoma Experiencing Progression With Single-Agent BRAF Inhibitor. *J. Clin. Onc.* 2014; 32:1–8.

19. Zhang C, Spevak W, Zhang Y, Burton EA, Ma Y, Habets G, Zhang J, Lin J, Ewing T, Matusow B, Tsang G, Marimuthu A, Cho H, Wu G, Wang W, Fong D, Nguyen H, Shi S, Womack P, Nespi M, Shellooe R, Carias H, Powell B, Light E, Sanftner L, Walters J, Tsai J, West BL, Visor G, Rezaei H, Lin PS, Nolop K, Ibrahim PN, Hirth P, Bollag G. RAF Inhibitors That Evade Paradoxical MAPK Pathway Activation. *Nature*. 2015; 526:583–586. [PubMed: 26466569]
20. Weber CK, Slupsky JR, Kalmes HA, Rapp UR. Active RAS Induces Heterodimerization of CRAF and BRAF. *Cancer Res*. 2001; 61:3595–3598. [PubMed: 11325826]
21. Rushworth LK, Hindley AD, O'Neill E, Kolch W. Regulation and Role of RAF-1/B-RAF Heterodimerization. *Mol. Cell. Biol*. 2006; 26(6):2262–2272. [PubMed: 16508002]
22. Lavoie H, Thevakumaran N, Gavory G, Li JJ, Padeganeh A, Guiral S, Duchaine J, Mao DY, Bouvier M, Sicheri F, Therrien M. Inhibitors that stabilize a closed RAF kinase domain conformation induce dimerization. *Nat. Chem. Biol*. 2013; 9(7):428–436. [PubMed: 23685672]
23. Villanueva J, Vultur A, Herlyn M. Resistance to BRAF Inhibitors: Unraveling Mechanisms and Future Treatment Options. *Cancer Res*. 2011; 71:7137–7140. [PubMed: 22131348]
24. Roring M, Herr R, Fiala G, Heilmann K, Braun S, Eisenhardt AE, Halbach S, Capper D, von Deimling A, Schamel WW, Saunders DN, Brummer T. Distinct Requirement for an Intact Dimer Interface in Wild-Type, V600E and Kinase-Dead BRAF Signalling. *EMBO Journal*. 2012; 31(11):2629–2647. [PubMed: 22510884]
25. Freeman AK, Ritt DA, Morrison DK. Effects of Raf Dimerization and Its Inhibition on Normal and Disease-Associated Raf Signaling. *Mol. Cell*. 2013; 49:751–758. [PubMed: 23352452]
26. Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, Chen Z, Lee MK, Attar N, Sazegar H, Chodon T, Nelson SF, McArthur G, Sosman JA, Ribas A, Lo RS. Melanomas Acquire Resistance to BRAF(V600E) Inhibition by RTK or N-RAS Upregulation. *Nature*. 2010; 468(7326):973–977. [PubMed: 21107323]
27. Poulikakos PI, Zhang C, Bollag G, Shokat KM, Rosen N. RAF inhibitors transactivate RAF dimers and ERK signaling in cells with wild-type BRAF. *Nature*. 2010; 464(7287):427–430. [PubMed: 20179705]
28. Sievert AJ, Lang S-S, Boucher KL, Madsen PJ, Slaunwhite E, Choudhary N, Kellet M, Storm PB, Resnick AC. Paradoxical activation and RAF inhibitor resistance of BRAF protein kinase fusions characterizing pediatric astrocytomas. *PNAS*. 2013; 110(15):5957–5962. [PubMed: 23533272]
29. Sosman JA, Kim KB, Schuchter L, Gonzalez R, Pavlick AC, Weber JS, McArthur GA, Hutson TE, Moschos SJ, Flaherty KT, Hersey P, Kefford R, Lawrence D, Puzanov I, Lewis KD, Amaravadi RK, Chmielowski B, Lawrence HJ, Shyr Y, Ye F, Li J, Nolop KB, Lee RJ, Joe AK, Ribas A. Survival in BRAF V600-Mutant Advanced Melanoma Treated With Vemurafenib. *N. Engl. J. Med*. 2012; 366(8):707–714. [PubMed: 22356324]
30. Rajakulendran T, Sahmi M, Lefrançois M, Sicheri F, Therrien M. A dimerization-dependent mechanism drives RAF catalytic activation. *Nature*. 2009; 461(7263):542–545. [PubMed: 19727074]
31. Thevakumaran N, Lavoie H, Critton DA, Tebben A, Marinier A, Sicheri F, Therrien M. Crystal structure of a BRAF kinase domain monomer explains basis for allosteric regulation. *Nat. Struct. Mol. Biol*. 2014; 22(1):37–44. [PubMed: 25437913]
32. Qin J, Xie P, Ventocilla C, Zhou G, Vultur A, Chen Q, Liu Q, Herlyn M, Winkler J, Marmorstein R. Identification of a Novel Family of braf-V600E Inhibitors. *J. Med. Chem*. 2012; 55:5220–5230. [PubMed: 22537109]
33. Zhang R, Monsma F. Fluorescence-based Thermal Shift Assays. *Curr. Opin. Drug Disc*. 2010; 13:389–402.
34. Otwinowski, Z.; Minor, W. Processing of X-ray Diffraction Data Collected in Oscillation Mode. In: Carter, CW., Jr; Sweet, RM., editors. *Method Enzymol.*, Volume 276: Macromolecular Crystallography, part A. Vol. 276. New York: Academic Press; 1997. p. 307–326.
35. Kabsch W. XDS. *Acta Cryst*. 2010; D66:125–132.
36. Adams PD, Afonine PV, Bunkóczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung L-W, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr*. 2010; D66:213–221.

37. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. *J. Appl. Cryst.* 2007; 40:658–674. [PubMed: 19461840]
38. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and Development of Coot. *Acta Crystallogr.* 2010; D66:486–501.
39. Afonine PV, Grosse-Kunstleve RW, Echols N, Headd JJ, Moriarty NW, Mustyakimov M, Terwilliger TC, Urzhumtsev A, Zwart PH, Adams PD. Towards automated crystallographic structure refinement with phenix.refine. *Acta Cryst.* 2012; D68:352–367.
40. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Adv. Drug Deliv. Rev.* 2001; 46:3–26. [PubMed: 11259830]
41. Illendula A, Pulikkan JA, Zong H, Grembecka J, Xue L, Sen S, Zhou Y, Boulton A, Kuntimaddi A, Gao Y, Rajewski RA, Guzman ML, Castilla LH, Bushweller JH. A Small-Molecule Inhibitor of the Aberrant Transcription Factor CBF β -SMMHC Delays Leukemia in Mice. *Science.* 2015; 347(6223):779–784. [PubMed: 25678665]
42. Hu-Lieskovan S, Mok S, Moreno BH, Tsoi J, Robert L, Goedert L, Pinheiro EM, Koya RC, Graeber TG, Comin-Anduix B, Ribas A. Improved Antitumor Activity of Immunotherapy with BRAF and MEK Inhibitors in BRAF^{V600E} Melanoma. *Sci. Transl. Med.* 2015; 7(129):279–241.
43. Moriarty NW, Grosse-Kunstleve RW, Adams PD. electronic Ligand Builder and Optimization Workbench (eLBOW): a tool for ligand coordinate and restraint generation. *Acta Cryst.* 2009; D65:1074–1080.
44. Chen VB, Arendall WB, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, Richardson DC. MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Cryst.* 2010; D66:16–21.
45. Villanueva J, Vultur A, Lee JT, Somasundaram R, Fukunaga-Kalabis M, Cipolla AK, Wubbenhorst B, Xu X, Gimotty PA, Kee D, Santiago-Walker AE, Letrero R, D'Andrea K, Pushparaian A, Hayden JE, Brown KD, Laquerre S, McArthur GA, Sosman JA, Nathanson KL, Herlyn M. Acquired Resistance to BRAF Inhibitors Mediated by a RAF Kinase Switch in Melanoma Can be Overcome by Co-Targeting MEK and IGF-1R/P13K. *Cancer Cell.* 2010; 18(6):683–695. [PubMed: 21156289]
46. Hildbrand S, Mair H, Nikolaev R, Ren Y, Wright AJ. Process for the Manufacture of Pharmaceutically Active Compounds. U.S. Patent WO20110028511. 2011 Feb 3.
47. Bono, F.; Guillo, N.; Maffrand, JP.; Fons, P.; Olsen, JA.; Gilles, A. Preparation of FGF receptor agonist dimeric compounds, particularly bisindolizines, bisimidazo[1,5-a]pyridines and their derivatives. FR Patent. WO2007080325.
48. Hu EC, Stites H, Yu EA, Germino HS, Meharena PJ, Stork AP, Kornev SS, Taylor AS. Shaw. *Cell.* 2013; 154:1036–1046. [PubMed: 23993095]

Figure 1. Structure of BRAF kinase domain and rational for linked vemurafenib BRAF inhibitors

(a) Structure of the BRAF^{WT} kinase domain crystallized with Sorafenib (accession code 1UWH). The αC-Helix and R509 residue are highlighted in orange. (b) Close up of BRAF dimer interactions mediated by R509. (c) Initial scaffold for vemurafenib linked inhibitors where n equals the number of polyethylene glycol moiety groups.

Figure 2. Potency of first generation linked vemurafenib inhibitors

(a) Dose response curves of the Vem-Vem class of linked inhibitors against BRAF^{V600E} with unlinked PLX4720 (PLX) as a control. Calculated IC₅₀ values are indicated. The data is the average of two separate experiments, each performed in duplicate with $\pm$ SEM shown. 95% Confidence Intervals are: PLX (75.8 nM to 195 nM), Vem-6-Vem (97.8 nM to 291 nM), Vem-5-Vem (111 nM to 356 nM), Vem-4-Vem (114 nM to 295 nM), Vem-3-Vem (186 nM to 364 nM), and Vem-2-Vem (391 nM to 994 nM). (b) Dose response curves of Vem-6-Vem and Vem-3-Vem against BRAF^{WT} with PLX as a control, carried out in triplicate for PLX and Vem-6-Vem and in duplicate for Vem-3-Vem with $\pm$ SEM shown. The 95% Confidence Intervals are: PLX (335 nM to 883 nM), Vem-6-Vem (1.87 μM to 8.75 μM) and Vem-3-Vem (1.69 μM to 4.50 μM). (c) Thermal shift assay measurements of melting temperature for BRAF^{V600E} with no inhibitor, and with 50 μM of Vem-6-Vem, Vem-3-Vem, Vem-2-Vem, and PLX as a control, with calculated melting temperatures listed (n=6) with $\pm$ SEM shown. 95% Confidence Intervals are as follows: DMSO Control (35.4 °C to 36.2 °C), PLX (54.0 °C to 55.1 °C), Vem-6-Vem (43.7 °C to 45.0 °C), Vem-3-Vem (39.1 °C to 45.0 °C), and Vem-2-Vem (36.5 °C to 37.1 °C). (d) BRAF^{V600E} (WM983B cell line, solid

line) and BRAF^{WT} (WM3000, dashed line) cell lines were treated with the indicated doses of Vem-6-Vem or Vem-3-Vem for 72h. Cell viability was determined by MTT assays and calculated relative to DMSO-treated cells. Data show average cell viability (n=7) +/- SEM.

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Figure 3. Structure of BRAF^{V600E}/Vem-6-Vem complex

(a) Structure of BRAF^{V600E} bound to Vem-6-Vem. The αC-helix and R509 residues are highlighted in orange. (b) Overlay of one subunit of the BRAF^{V600E}/Vem-6-Vem dimer with an active dimer conformation (accession code 1UWH). (c) Superposition of BRAF^{V600E}/inhibitor structures (indicated by color-code) highlighting the position of the αC-helix and presence or absence of the AS-H1 helix. (d) Comparison of the αC-helix in active and inactive conformations highlighting the disposition of key residues, shown as a stereoimage.

Figure 4. Structure of BRAF^{V600E}/Vem-3-Vem complex

(a) Overlay of BRAF^{V600E}/Vem-6-Vem (green) and BRAF^{V600E}/Vem-3-Vem (cyan) structures. (b) Close-up of Vem-3-Vem inhibitor (magenta) and the simulated annealing omit map in green contoured at 2.5 sigma. (c) Close up of BRAF^{V600E} residues Q461 and H539 (purple) highlighting their proximity to the Vem-3-Vem linker (pink), shown as a stereo image.

Figure 5. Development of second generation linked vemurafenib inhibitors

(a) Second generation linked vemurafenib inhibitors utilizing bis-amide linkers. (b) Dose response curves of second generation inhibitors against BRAF^{V600E} with unlinked PLX4720 (PLX) as a control. Calculated IC₅₀ values are indicated. The data is the average of two separate experiments, each performed in duplicate with $\pm$ SEM shown. 95% Confidence Intervals are: PLX (70.0 nM to 191 nM), Vem-BisAmide-1 (2.28 μM to 5.75 μM), Vem-BisAmide-2 (23.7 nM to 47.5 nM), Vem-BisAmide-3 (114 nM to 332 nM), and Vem-3-Vem (165 nM to 352 nM) (c) Thermal stability assay of linked vemurafenib inhibitors with

calculated melting temperatures listed (n=6) with $\pm$ SEM shown. 95% Confidence Intervals are as follows: DMSO Control (35.5 °C to 36.3 °C), PLX (54.0 °C to 55.1 °C), Vem-6-Vem (43.7 °C to 45.0 °C), and Vem-BisAmide-2 (52.0 °C to 53.3 °C),

Figure 6. Structure of BRAF^{V600E}/Vem-BisAmide-2 complex and functional characterization of Vem-BisAmide-2

(a) Structure of BRAF^{V600E} (violet) bound to Vem-BisAmide-2 (pink) with a simulated annealing omit map in green contoured at 2.5 sigma, highlighting close proximity between the carbonyls of the linker amide and Q461, shown as a stereo image (b) Dose response curves of Vem-BisAmide-2 against BRAF^{V600E} (blue) and BRAF^{V600E/Q461A} (cyan) with PLX4720 (PLX) against both proteins as a control. Calculated IC₅₀ values from duplicate measurements are indicated with $\pm$ SEM shown. 95% Confidence Intervals are as follows: BRAF^{V600E}/Vem-BisAmide-2 (29.8 nM to 106 nM), BRAF^{V600E/Q461A}/Vem-BisAmide-2

(286 nM to 755 nM), BRAF^{V600E}/PLX (83.8 nM to 184 nM), and BRAF^{V600E/Q461A}/PLX (54.7 nM to 108 nM). (c) Sedimentation Velocity of BRAF^{V600E} (blue) and BRAF^{V600E/R509H} (red) as a function of added Vem-BisAmide-2. (d) Log plots of Sedimentation Equilibrium experiments showing theoretical monomer (red) and theoretical dimer (blue) slopes compared to BRAF^{V600E/R509H} with equimolar concentrations of protein and Vem-BisAmide-2 (purple) and PLX (cyan).

Figure 7. Activity of Vem-BisAmide-2 against melanoma cell lines

(a-b) Effect of Vem-3-Vem (cyan), Vem (black), Vem-BisAmide-2 (blue) and Vem-BisAmide-1 (pink) on viability of BRAF^{V600E} (WM983B; a) and BRAF^{WT} (WM3000; b) melanoma cell lines treated as in figure 2. Data show Average cell viability from 3 independent experiments (n=7) $\pm$ SEM. (c) WM983B (top) and WM3000 (bottom) melanoma cells were treated with the indicated compounds for 18 hr. Total protein lysates were analyzed by immunoblotting using antibodies against phospho-ERK and phospho-

MEK. Actin was used as a loading control. Quantification of bands were performed using Odyssey software (Licor) and normalized to the DMSO treatment band.

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Table 1

Summary of Crystallographic Statistics

Crystal	BRAF ^{V600E} / Vem-6-Vem	BRAF ^{V600E} / Vem-3-Vem	BRAF ^{V600E} / Vem-BisAmide-2
Resolution Range (Å)	29.21–2.29 (2.34–2.29)	138.00–2.19 (2.24–2.19)	50–2.70 (2.75–2.70)
Space Group	P2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit Cell (a, b, c, α, β, γ)	56.84, 67.75, 67.88 90, 90.32, 90	64.61, 68.26, 276.01 90, 90, 90	65.10, 68.44, 275.60 90, 90, 90
Total Reflections	59,386	82,6367	192,419
Unique Reflections	22,352	63,307	34,634
Redundancy	2.7(2.7)	12.9 (12.100)	5.5 (5.8)
Completeness (%)	95.31 (90.21)	98.94 (96.12)	99.72 (99.50)
Mean I/sigma (I)	13.19 (2.76)	15.64 (1.37)	16.23 (2.62)
Wilson B Factor	32.72	51.44	57.32
R-merge	0.097 (0.521)	0.104 (2.323)	0.110 (0.862)
R-work	0.1947	0.2072	0.2122
R-free	0.2538	0.2597	0.2729
RMS (bonds)	0.008	0.009	0.009
RMS (angles)	1.18	1.19	1.28
Ramachandran Favored (%)	95	96	96
Ramachandran Outliers (%)	0.41	0.39	0.42
Clashscore	9.50	6.31	6.22
Average B Factor	27.20	50.10	41.10

Numbers in parentheses are for the last resolution shell.