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Development of Allosteric BRAF Peptide Inhibitors Targeting the Dimer Interface of BRAF

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Abstract

BRAF is the most frequently mutated kinase in human cancers and is one of the major effectors of oncogenic RAS, making BRAF a target of considerable interest for anti-cancer drug development. Wild-type BRAF and a variety of oncogenic BRAF mutants are dependent on dimerization of the kinase domain, which also emerges as a culprit of drug resistance and side effects of current BRAF therapies. Thus, allosteric BRAF inhibitors capable of disrupting BRAF dimers could abrogate hyperactivated MAPK (mitogen-activated protein kinase) signaling driven by oncogenic BRAF or RAS and overcome the major limitations of current BRAF inhibitors. To establish this, we applied an *in silico* approach to design a series of peptide inhibitors targeting the dimer interface of BRAF. One resulting inhibitor was found to potently inhibit the kinase activity of BRAF homo- and heterodimers, including oncogenic $BRAF^{G469A}$ mutant. Moreover, this inhibitor synergizes with FDA-approved, ATP-competitive BRAF inhibitors against dimeric BRAF, suggesting that allosteric BRAF inhibitors have great potential to extend the application of current BRAF therapies. Additionally, targeting the dimer interface of BRAF kinase leads to protein degradation of both RAF and MEK, uncovering a novel scaffolding function of RAF in protecting large MAPK complexes from protein degradation. In conclusion, we have developed a potent lead peptide inhibitor for targeting the dimer interface of BRAF in cancer cells. The dual function of this peptide inhibitor validates the strategy for developing allosteric BRAF inhibitors that specifically dissociate RAF dimers and destabilize the MAPK signaling complex.

Graphical abstract

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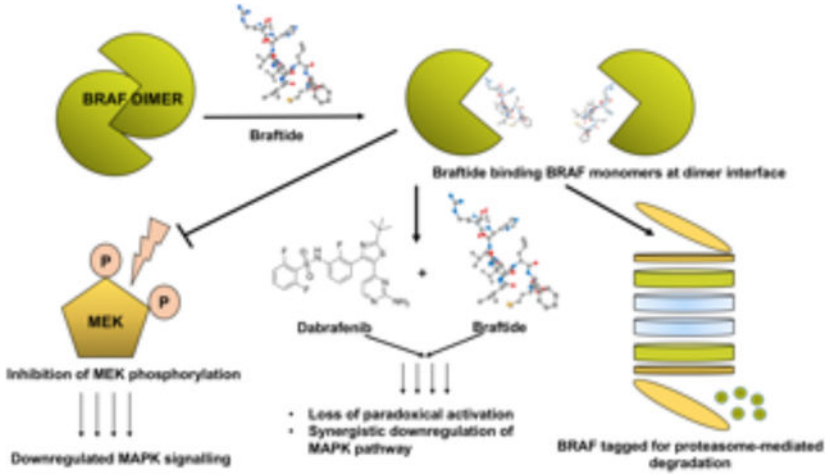

BRAF, together with its two isoforms ARAF and CRAF, belongs to the family of RAF kinases, which is a core component of the RAS/RAF/MEK/ERK signaling cascade, also called the mitogen-activated protein kinase (MAPK) cascade.(1, 2) MAPK cascade mediates signals from cell surface receptors to the nucleus to control vital cellular processes such as cell proliferation and differentiation. Oncogenic mutations in RAS or BRAF induce hyperactivation of MAPK signaling and subsequent tumorigenesis, making this cascade a target of considerable interest for anti-cancer drug development.(3, 4, 5) However, targeting RAS protein has been unsuccessful despite decades of efforts. As the major RAS downstream effector, BRAF is the most successful drug target among the core components of the MAPK cascade. Tumor cells possessing hyperactive MAPK signaling can be sensitized to apoptosis through selective inhibition of BRAF.(6)

There has been an intense effort to develop inhibitors for BRAF, which has led to two FDA-approved inhibitors, dabrafenib and vemurafenib. These ATP-competitive inhibitors potently inhibit the most common BRAF variant, V600E, which is present in the activation loop of the kinase. Vemurafenib and dabrafenib yield unprecedented response rates in melanoma patients harboring the V600E BRAF mutation.(7) Unexpectedly, they stimulate the same pathway in tumor cells containing wild-type BRAF and oncogenic RAS to induce secondary malignancies, a phenomenon known as ‘paradoxical activation’.(8, 9, 10) Moreover, their efficacy is only limited to $BRAF^{V600E}$ tumors while tumors carrying non-V600 BRAF mutations display intrinsic drug resistance.(11) These concerns surrounding current BRAF therapies underscore the urgent need for development of alternative therapeutic strategies. Non-V600 mutations constitute approximately 50% of BRAF mutations in lung cancer and RAS mutations occur in 30% of cancer patients(12), suggesting that a substantial number of cancer patients could benefit from novel therapies targeting BRAF.

Previous studies(11, 13) support that, distinct from $BRAF^{V600E}$ which functions as a monomer, both wild-type BRAF and non-V600 BRAF mutants require an intact dimer interface (DIF) to be functional. BRAF DIF is present in the kinase domain of BRAF at the tail end of the α-C helix.(13) It spans ~20 residues (aa 501–520), with R509 being the central residue that is critical for dimer integrity.(14) RAF dimerization is stabilized by

mostly a hydrogen bond network involving R509, L515, and M517. It has been shown the triple mutation, R509/L515/M517, completely abolishes the kinase activity of wild-type BRAF.(15) Furthermore, side effects of current BRAF inhibitors, including drug resistance and paradoxical activation, are contingent on the same DIF.(16) Many of the ATP-competitive inhibitors promote RAF dimerization in a RAS-dependent manner.(8, 17, 18) We thus hypothesize that allosteric inhibitors capable of disrupting the DIF of BRAF could abrogate hyperactivated MAPK signaling driven by non-V600 BRAF mutations or RAS mutations while overcoming the major limitations of current BRAF inhibitors. This DIF region is conserved across the RAF family members, but not in other protein kinases, therefore such inhibitors may achieve higher specificity towards RAF, in comparison with ATP-competitive inhibitors.

Here, we report a 10-mer peptide inhibitor braftide, that is designed using a computational approach to block RAF dimerization. *In vitro* kinase assays with purified full-length wild-type BRAF and $BRAF^{G469A}$ demonstrate that braftide potently inhibits $BRAF^{G469A}$ and inhibits $BRAF^{WT}$ to a lesser extent. Other than abolishing the kinase activity of dimeric BRAF, this inhibitor triggers selective protein degradation of BRAF and MEK through proteasome-mediated protein degradation in cells. The dual mechanism of inhibition, inducing degradation and inhibition of kinase activity, makes this peptide a more potent inhibitor, which was verified by cell viability assays in KRAS mutant tumor cells. Additionally, we observed that the combination of ATP-competitive inhibitors and braftide eliminates paradoxical activation, suggesting an alternative strategy to improve the efficacy of current ATP-competitive inhibitors. Together, our work establishes the RAF dimer interface as a promising therapeutic target.

RESULTS AND DISCUSSION

Computational Peptide Design Targeting the DIF of BRAF.

Structural analyses of dimeric BRAF reveal key features of the DIF, which provide an excellent starting point for designing inhibitors targeting this interface.(14) As the first step to identify peptide inhibitors targeting this dimer interface, one on-state structure of BRAF dimer (PDB ID: 1UWH(19)) and one off-state structure (PDB ID: 3TV6(20)) were subject to PeptiDerive analysis. PeptiDerive systematically isolates peptides from one designated protein chain using a sliding window and then evaluates the contributions of these peptide segments to the overall dimer interaction individually. From PeptiDerive analyses, six peptide sequences with the highest Relative Interface Score (%) were identified (Supplementary Table 1).

Evaluation of the Peptide Inhibitors against Wild-type BRAF and Oncogenic $BRAF^{G469A}$.

Since the activation of wild-type BRAF and oncogenic non-V600 BRAF mutants is dependent on an intact dimer interface(13), peptides potently disrupting BRAF dimers function as allosteric BRAF inhibitors. We evaluated the inhibition potency of the identified peptides by quantifying the kinase activity of full-length (FL) $BRAF^{WT/G469A}$ *via* ELISA assay(21), in which phosphorylated MEK was probed. Different from the isolated catalytic domain which is dominantly monomeric in solution, FL-BRAF protein purified from

HEK293F cells adopts an active dimeric configuration in solution(21), therefore it is advantageous to evaluate dimer breaker inhibitors using FL-BRAF purified from HEK293F cells. The G469A mutation was chosen as the representative of non-V600 mutations, as it is the most prevalent non-V600 BRAF mutation identified in lung cancer.(22) In addition, it has been demonstrated that the G469A variant is dependent on the integrity of the dimer interface.(23)

One 10-mer peptide, whose sequence (TRHVNILLFM) is derived from the dimer interface of BRAF, was named as braftide. Upon computational docking, braftide was predicted to bind predominantly at the dimer interface of BRAF kinase domain, in which multiple interactions were projected between braftide and BRAF (Fig. 1A&B). The Arg residue (R2) of braftide forms hydrophobic interactions with W450 and hydrogen bonds with T508 and F516 residues of BRAF.

In addition, the Thr (T1) and His (H3) residues of braftide form hydrogen bonds with R509 and H477 residues of BRAF, respectively. The predicted interactions between braftide and BRAF mirror the corresponding interactions between the two BRAF protomers observed in crystal structures (Supplementary Fig. 1).

Among all the peptides evaluated (Supplementary Table 2 & Supplementary Fig. 2A–C), braftide showed the most potent inhibition, with IC_{50} values of 364 nM against wild-type BRAF and 172 nM against oncogenic $BRAF^{G469A}$, (Fig. 1C). As the R509H mutation has been well-established to disrupt BRAF dimers, we expect that mutating R2 to His residue will result in destabilized protein-braftide interactions. We mutated R2 to His residue and generated a peptide, named as R/H-braftide (T**H**HVNILLFM). As shown in Fig. 1C, R/H-braftide curves demonstrated a right shift to those for braftide, with increased IC_{50} values of 1.5 μM (~ 4-fold increase) and 2.5 μM (~ 15-fold increase) for wild-type and G469A respectively. The reduced inhibition potency of the R/H-braftide against BRAF, suggests that R2 is involved in peptide/protein interaction, as predicted by the model (Fig. 1A). We synthesized a Cy3-tagged braftide and evaluated its binding affinity toward the catalytic domain of BRAF (BRAF-WT-KD) (Fig. 1D). We switched to the kinase domain for this binding assay, as the yield of full-length BRAF is too low to carry out this experiment. Although the measured K_d (6.69 μM) may not reflect the actual binding affinity between braftide and full-length BRAF, it clearly verified the direct interaction between braftide and the kinase domain of BRAF.

Next, we evaluated whether the observed inhibition derives from the ability of braftide to disrupt BRAF homodimers. In the co-immunoprecipitation experiment, FLAG-tagged BRAF and V5-tagged BRAF were co-expressed in HEK293 cells. Cell lysate was subject to Flag antibody-conjugated resin, which was later probed for V5-tagged BRAF. As shown in Fig. 1E, adding 50 μM of braftide to cell lysate decreased the formation of BRAF homodimers, consistent with our hypothesis that braftide disrupts BRAF dimers. As compared with the IC_{50} value obtained from purified FL-BRAF, a relatively higher concentration of braftide was applied to disrupt cellular BRAF dimers overexpressed in HEK293 cells. We believe that the scaffolding function of other proteins in cell lysate might contribute to a BRAF dimer that is more resistant to dimer breakers. In addition, it is

possible that the peptide was degraded during incubation with cell lysate, although protease inhibitors were added to the cell lysate. Overall, our data suggest that braftide allosterically inhibits BRAF activation by blocking formation of the BRAF dimer.

Delivery of Braftide into HEK293 cells Decreases MAPK Signaling.

Braftide was conjugated with the TAT sequence (GRKKRRQRRRPQ), a cell-penetrating peptide widely used in peptide drugs.(24) *In vitro* enzyme assays demonstrate that the addition of TAT sequence does not jeopardize the inhibition potency of braftide (Supplementary Fig. 2D). Instead, it increases the inhibition potency of braftide, with an IC_{50} value of 43 nM. One possibility is that the addition of extra amino acids facilitates the folding of the 10-mer peptide, making it a more potent dimer breaker. HEK293 cells were transiently transfected with plasmid encoding either wild-type BRAF or $BRAF^{G469A}$. 48 hr post-transfection, the cells were treated with various concentrations of TAT-braftide for 4hr. In parallel, TAT peptide was used as the negative control. The activity of BRAF was quantified by probing for phospho-MEK1/2. Actin is used as the loading control. Consistent with the *in vitro* kinase assays, treatment with TAT-braftide significantly reduced the activity of BRAF in a dose-dependent manner (Fig. 2A). Since no inhibition effect was observed for TAT control peptide in transiently transfected HEK293 cells, the decreased pMEK is caused by braftide, not TAT tag (Fig. 2B). Similar to Fig. 1E, high micromolar concentration of braftide is required to effectively diminish MAPK signaling, suggesting that either braftide has been degraded upon delivery inside cells or cellular BRAF dimers are more resistant to braftide.

Braftide Triggers Protein Degradation of BRAF and MEK.

Intriguingly, the protein levels of overexpressed BRAF and endogenous MEK1 were markedly decreased in a dose-dependent manner upon TAT-braftide treatment (Fig. 2A). Previously we have found that dimerization of RAF significantly augments the expression level of RAF proteins in HEK293 cells (Supplementary Fig. 3).(25) These observations led us to hypothesize that TAT-braftide not only exerts an inhibitory effect but also triggers proteolysis of BRAF and MEK by disrupting BRAF dimerization in HEK293 cells. In light of this, we pre-treated HEK293 cells with proteasome inhibitor bortezomib before adding TAT-braftide and probed for total BRAF, total MEK, and phosphorylated MEK (Fig. 3A). Three independent experiments were summarized in Fig. 3B. Although proteasome inhibition rescues BRAF from protein degradation, TAT-braftide still successfully inhibited the kinase activity of BRAF, suggesting that the diminished MAPK signaling upon braftide treatment is due to the dual function of braftide: inhibiting the kinase activity of BRAF while inducing proteasome-mediated protein degradation, the latter reflects the non-catalytic function of BRAF. To evaluate the potent induction of degradation, we compared the half-life of BRAF protein in the presence and absence of braftide. HEK293 cells transiently transfected with wild-type BRAF were treated with cycloheximide. Braftide treatment reduced the half-life of BRAF from > 10 h to ~ 2h (Fig. 3 C&D). Together, our data support that disruption of the dimerization interface with a peptide inhibitor sequesters BRAF in an inactive state to induce degradation of the MAPK complex.

Braftide is Potent Against BRAF/CRAF Heterodimers and p61 BRAF^{V600E} Dimers.

The BRAF/CRAF heterodimer has been identified as the most active RAF dimer.(26) Formation of BRAF/CRAF dimers is the major culprit of drug resistance and paradoxical activation, two major limitations of current BRAF drugs.(11) We did BLAST on the sequence of the dimer interface of BRAF (aa503–520 of BRAF) and found that the sequence is only conserved among the RAF kinase family and KSR1/2 pseudokinases (Fig. 4A), which is also supported by the available BRAF and CRAF structures.(27, 8) In light of this, we evaluated the inhibition potential of braftide against BRAF/CRAF heterodimers overexpressed in HEK293 cells. Co-IP experiments support that BRAF and CRAF form heterodimers under the same conditions (Supplementary Fig. 4). Upon braftide treatment, both BRAF and CRAF were degraded together with diminished MAPK signaling (Fig. 4B), suggesting that the BRAF/CRAF heterodimer is sensitive to braftide as well.

We also investigated the activity of TAT-braftide on p61 BRAF^{V600E} (Fig. 4C) and BRAF^{V600E} (Fig. 4D). p61 BRAF^{V600E} is an aberrantly spliced version of BRAF that is approximately 61 kDa in size. p61 shows constitutive dimerization capability in cell lines and has previously been shown to be one common mechanism by which BRAF^{V600E} melanoma patients acquire resistance to ATP-competitive inhibitors.(28) Spliced BRAF^{V600E} is a RAS-independent dimer but can signal ERK either as a monomer or dimer. We transiently transfected p61-BRAF^{V600E} in HEK293 cells and treated them with increasing concentrations of TAT-braftide. We observed a decrease in both pMEK and pERK levels consistent with downregulated MAPK signaling, although the protein level of p61 was not affected as dramatically as wild-type BRAF (Fig. 4C). Conversely, braftide inhibits BRAF^{V600E} to a much lesser extent (Fig. 4D), suggesting that p61 and BRAF^{V600E} have distinct activation mechanisms.

Evaluation of the Synergy between Braftide and ATP-Competitive BRAF Inhibitors.

The efficacy of current ATP-competitive BRAF inhibitors is limited to BRAF^{V600E}.(28) Inhibition of wild-type BRAF dimers by dabrafenib and vemurafenib is limited by induction of negative cooperativity within a dimer in which the inhibitor-bound protomer allosterically activates the inhibitor-free protomer to cause drug resistance and paradoxical activation.(29, 17) Braftide is designed to dissociate BRAF dimers, thus we hypothesize that braftide might synergize with ATP-competitive inhibitors to potently inhibit dimeric BRAF, expanding the application of current BRAF therapies to all BRAF variants. We evaluated the IC_{50} values of dabrafenib against purified FL-BRAF in the presence and absence of braftide. Although paradoxical activation was not recapitulated using purified kinase domain, we previously demonstrated that FL-BRAF was paradoxically activated by dabrafenib and vemurafenib *in vitro*(21), which makes FL-BRAF a valuable tool to evaluate paradox breakers. As shown in Fig. 5A, the IC_{50} value of dabrafenib against dimeric wild-type BRAF was decreased significantly after pre-incubating BRAF with 70 nM of braftide, strongly supporting our hypothesis. Moreover, the notorious ‘paradoxical activation’ caused by dabrafenib monotherapy at subsaturating concentrations was abolished by braftide (Fig. 5A). In the presence of much lower concentration of braftide (10 nM), we observed that the IC_{50} value of dabrafenib against BRAF^{G469A} was decreased from ~ 5 nM to 0.1 nM (50-fold decrease) and that the paradoxical activation was eliminated by braftide (Fig. 5B). In parallel, we

performed a dose-response of braftide with fixed concentrations of dabrafenib (0.1 nM for wild-type and 1 nM for G469A). As shown in Supplemental Fig. 5, the addition of dabrafenib didn't cause a significant change of IC_{50} value, suggesting that dabrafenib binding to the active site has little effect on the affinity of BRAF dimer under tested conditions.

The synergy effect was further evaluated in HEK293 cells overexpressing either wild-type BRAF or $BRAF^{G469A}$. As shown in Fig. 5C, dabrafenib alone activated the MAPK signaling at lower concentrations and only performed as an inhibitor at concentrations above 10 μM, validating that dabrafenib could not potently inhibit BRAF homodimers because it triggers paradoxical activation.(30) Pre-treatment of HEK293 cells with 75 μM of TAT-braftide for 2 h abrogated the paradoxical effect caused by lower doses of dabrafenib. A similar pattern was observed for $BRAF^{G469A}$ (Fig. 5D). Intriguingly, dabrafenib more significantly activated both MAPK signaling in HEK293 cells expressing $BRAF^{G469A}$ and purified FL-$BRAF^{G469A}$ (Fig. 5D), demonstrating that extra caution should be taken when targeting non-V600 BRAF mutants with ATP-competitive inhibitors. On the other hand, disruption of the dimer interface of BRAF proves to be a very promising strategy to eliminate the major drawbacks of ATP-competitive inhibitors. The same synergy effect between vemurafenib and braftide was validated (Supplementary Fig. 6). Our experiments demonstrated that braftide and FDA-approved ATP-competitive inhibitors work in synergy to diminish paradoxical activation and sufficiently inhibit MAPK signaling in HEK293 cells.

Evaluation of Antiproliferative Activity of Braftide on Cancer Cells.

The dual mechanism of TAT-braftide inhibition on MAPK signaling was further explored in two cancer cell lines, HCT116 and HCT-15 (KRAS ^{G13D}-colon carcinoma cell lines). Hyperactivated KRAS markedly increases the levels of RAS-dependent BRAF homodimers and BRAF/CRAF heterodimers.(31, 32) Cell-based assays with TAT-braftide show inhibition of the MAPK pathway in the HCT116 cell line (Fig. 6A), as evidenced by diminished phospho-MEK and phospho-ERK, together with degradation of endogenous BRAF and MEK, with an IC_{50} value below 10 μM. We believe that the discrepancy between the IC_{50} values of braftide against HCT116 and HEK293 cells transiently transfected with FL-RAF is due to the protein level of RAF. The endogenous RAF protein level is at least 100-fold lower than that of overexpressed RAF in HEK293 cells (data not shown). Since ATP-competitive BRAF inhibitors have been shown to cause tumor cell death primarily through apoptosis rather than necrosis(6), we evaluated the apoptotic activity of TAT-braftide on the two cancer cell lines via cell viability assays (Fig. 6B&C). TAT-braftide treatment caused dose-dependent inhibition of cell growth of HCT116 and HCT-15 cells, with EC_{50} values of 7.1 μM and 6.6 μM, respectively, demonstrating potent inhibitory activity on cell viability in KRAS-mutated colon cancer cells. The TAT peptide was used as a negative control and no cell death was observed at concentrations up to 100 μM (Fig. 6B&C).

CONCLUSIONS

All RAF inhibitors developed to date belong to the ATP-competitive inhibitor. Structure-guided drug design led to the approval of vemurafenib and dabrafenib that preferentially

stabilize the 'αCOUT' configuration.[33] Unfortunately, they are ineffective against non-V600 BRAF mutant.(33, 34) 'αC-IN' inhibitors are designed to equally occupy both protomers of RAF dimers(35), therefore hold promise in dimeric BRAF-dependent tumors. However, paradoxical activation of MAPK signaling is a property of both 'αC-OUT' and 'αC-IN' inhibitors. The underlying mechanism is still in debate. Conversely, allosteric RAF inhibitors have been understudied. Freeman *et al.* reported a 19-mer peptide which was derived directly from the dimer interface of BRAF and demonstrated a potential to dissociate BRAF/CRAF dimerization.(13) With this rationale in mind, we sought to develop smaller peptides (5mer-11mer) targeting the same dimer interface. Structures of BRAF have identified the key properties of the RAF dimer interface, which provides us a solid foundation to design dimer breakers through *in silico* approaches. Our 10-mer braftide has demonstrated efficacy against $BRAF^{G469A}$, a representative of dimeric BRAF mutants. Braftide potently inhibits dimeric BRAF by eradicating both the catalytic and non-catalytic functions of BRAF. As a result, it successfully avoids negative cooperativity and paradoxical activation. Our work further verifies that the RAF dimer interface is a promising drug target against malignancies driven by dimeric BRAF mutants or RAS mutants.

Most importantly, we demonstrate that braftide treatment causes degradation of the MAPK complex. We have shown that this RAF degradation is mediated through the proteasome. Our results uncover a previously unrecognized function of dimerization: sequestering RAF proteins in a conformation that is less prone to proteasome-mediated protein degradation. Our finding has important implications. Targeted degradation of disease-causing proteins using proteolysis targeting chimeras (PROTACs) has emerged as a powerful strategy to combat cancer.(36, 37) Similar to PROTACs, braftide-triggered selective degradation of RAF and MEK could be advantageous over small molecule inhibitors. Other than inhibition of kinase activity, elimination of all functions of BRAF by protein degradation ensures a more complete inactivation of MAPK signaling. Moreover, the dual inhibition mechanism of braftide most likely circumvents reactivation of the same pathway and hence delays or prevents drug resistance by this mechanism, which is a common drawback of small molecule RAF inhibitors.(38) ATP-competitive inhibitors are identified to promote RAF dimerization.(39) It is reasonable to propose that this property may also enhance the half-life of RAF proteins, counteracting the efficacy of kinase inhibitors. Clearly, this feature should be considered when designing next generation of ATP-competitive inhibitors.

Structural analysis of various ATP-competitive RAF inhibitors suggest that the binding mode of an inhibitor can affect RAF dimerization[33]. Inhibitors that binds to the kinase domain in the 'αCOUT' configuration disfavors dimer formation, suggesting that this feature can be utilized to dissociate enzyme inhibition from paradoxical activation. In line with this concept, an analogue of vemurafenib, PLX8394, was recently reported to dissociate BRAF homo- and hetero-dimers.(40, 41) Development of ATP-competitive inhibitors that lead to further displacement of the αC helix is promising to completely ablate paradoxical activation, however, its efficacy towards a broad range of BRAF dimer-driven tumors has yet to be determined. Alternatively, concurrently targeting the dimer interface and the catalytic site with two types of inhibitors is effective to reduce paradoxical activation against dimeric BRAF, as supported by the combination treatment of braftide with vemurafenib and dabrafenib. Adding braftide to vemurafenib and dabrafenib proves to be a

successful strategy to target dimeric BRAF, with a potential to extend the current application of vemurafenib and dabrafenib to mutated RAS or non-V600E mutants.

Since dimerization is necessary for activation of all three RAF kinases, braftide is expected to behave more or less like pan-RAF inhibitors. Several of this type of inhibitors are currently under clinical trials. One major concern for pan-RAF inhibitors is that toxicity might arise from blocking wild-type RAF proteins in healthy tissue. Previous studies suggest that the three RAF kinases possess subtle difference in the dimer interface.(34, 39, 40) Consequently, they respond differently to RAF inhibitors regarding dimerization property. Similarly, the activation of non-V600 mutants depends largely on their dimerization capacity, which is different from wild-type BRAF. Designing potent inhibitors that are more sensitive to dimeric BRAF mutants might be feasible. A more definitive and detailed understanding of the mechanisms involved in regulation of different RAF dimers and BRAF variants is necessary to facilitate this approach.

Consistent with the notion that $BRAF^{V600E}$ can signal as a monomer, the effectiveness of braftide against $BRAF^{V600E}$ is dampened. Surprisingly, braftide inhibits ectopically expressed p61 $BRAF^{V600E}$, a spliced form of $BRAF^{V600E}$ that constitutively dimerizes in a RAS-independent manner.(28) Its dimerization feature confers intrinsic resistance to BRAF inhibition in $BRAF^{V600E}$ melanoma patients, however the kinase activity of p61 $BRAF^{V600E}$ is not contingent on the dimer interface. Hence, the observed inhibition effect of bradtide most likely stems from blocking the scaffolding function of p61 $BRAF^{V600E}$, rather than inhibiting the kinase activity of p61. In summary, our work provides a rationale to develop novel RAF inhibitors that evade negative cooperativity, paradoxical activation, and resistance mechanisms. Furthermore, such inhibitors would be a valuable chemical probe to dissect the biological significance of RAF dimerization in MAPK signaling.

While our manuscript is under review, Beneker *et. al.* (42) reported another series of linear and cyclic peptides whose sequences were derived from the same dimer interface where our braftide peptide was derived from. By performing detailed SAR studies, they concluded that cyclic peptide has a higher binding affinity to BRAF kinase domain. Together, the two independent studies complement each other and reiterate that the RAF dimer interface is a very promising drug target.

METHODS

Peptides

Peptides were purchased from Lifetein with TFA removal. Purity was determined through HPLC (>95%) and confirmed through Mass spectrometry.

Plasmids

6X-HIS-BRAF-WT/FLAG was prepared as previously described(43), 6X-HIS/BRAFG469A/FLAG and MBP-CRAF-FLAG were created using common cloning procedures with pcDNA™ 4/TO (Invitrogen) as the vector. 6X-HIS-BRAF-V600E/FLAG and 6X-HIS-BRAF-p61-V600E/FLAG were prepared similarly.

Computational peptide design

The sequence of the human serine/threonine-protein kinase B-raf (Accession Number: P15056) was obtained from the UniProKB database.(44) This sequence was taken as the query sequence for similarity search against the PDB database(45) using FASTA(46) at www.ebi.ac.uk. For the FASTA search, the parameter for both the Scores and Alignments was set to 2000. Next, for all the hits having >99% sequence identity with the query sequence, attempts were made to classify them as either on-state dimer structures or off-state dimer structures. This was done by cross checking them against the classified PDB list.(47) From the obtained two structure groups, one representing the on-state and one representing the off-state were chosen randomly. Finally, these two chosen structures were subjected to the ROSIE PeptiDerive searching engine(48) at http://rosie.rosettacommons.org/peptiderive respectively. Given a protein-protein complex structure, PeptiDerive identifies the linear peptide segment that contributes most to the protein-protein interaction. For each calculation, the receptor role was restricted to A chain and the partner role to B chain. The derived peptide length was set from 5 to 11. Based on the PeptiDerive results, linear and suggested cyclic peptides with the highest Relative Interface Scores were chosen for experimental validation.

Co-immunoprecipitation of BRAF homodimers with Braftide treatment

V5-tagged $BRAF^{WT}$ and FLAG-tagged $BRAF^{WT}$ were co-transfected in HEK293 cells for 48 hrs. Cells were harvested after this time and lysed in modified RIPA buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 0.1% NP40 (IGEPAL630), 1 mM EDTA, 5 % glycerol, 1 mM PMSF, 20 mM BGP, 2.5 mM Sodium Pyrophosphate, and 1 protease inhibitor tablet) and incubated with rotation for ~ 2 hrs at 4°C. Cell lysate (0.5 mg) was then treated with braftide (50 μM) for 90 min with rotation and then bound to FLAG-M2 magnetic resin. After few wash steps, the resin was resuspended in dilution buffer and analyzed through immunoblotting for co-immunoprecipitation FLAG and V5-tagged BRAF.

Cell Viability

Cell viabilities of HCT116 and HCT-15 cell lines were tested with treatment of TAT-braftide and the TAT control peptide. Cells were seeded onto poly-lysine coated, clear-bottomed, 96-well plates at ~15000 cells per well. After 24 hr, the cells were treated with TAT-braftide or the TAT control peptide at different concentrations (0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 μM). Upon 48 hr treatment, cell viability was determined through the WST cell viability assay. According to manufacturers' instructions, 10 μL of the WST reagent was added to each well and incubated at 37°C for 4 hrs. Absorbance readings for the plate were then taken at 450 nm in a Biotek plate reader.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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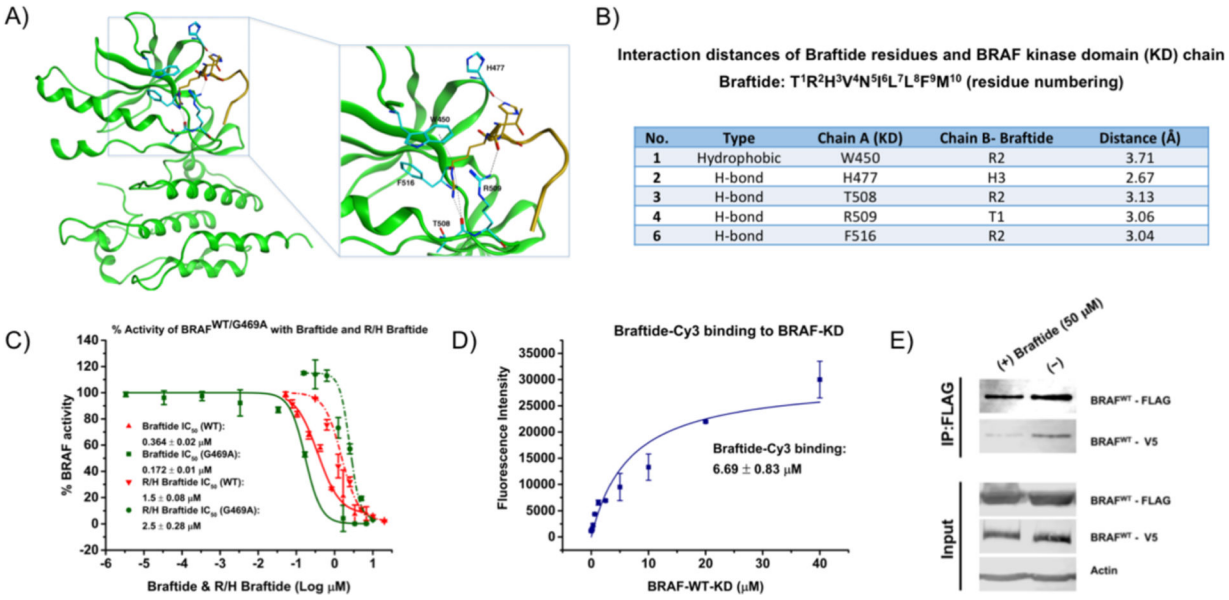

Figure 1. Braftide inhibits BRAF kinase activity by disrupting BRAF dimers.

A) Computational modeling of predicted binding of braftide to the dimer interface of the BRAF kinase domain (PDB:3TV6). Model was generated in Rosetta PeptiDerive and the amino acid interactions are highlighted. B) Predicted distances between the braftide residues and the residues of the BRAF kinase domain protein that contribute to relative interface scores. C) Dose-response curves generated from an ELISA for braftide and R/H-braftide against FL-BRAF$^{WT/G469A}$. 95% confidence intervals are: Braftide (WT: 0.213 μM to 1.65 μM, G469A: 0.003 μM to 0.419 μM) and R/H-Braftide (WT: 2.49 μM to 4.98 μM, G469A: 0.625 μM to 3.34 μM). Error bars represent standard deviations of triplicate measurements. IC50 values were obtained from dose-response curve (4-parameter logistic equation) function in Origin, from three independent experiments. D) Saturation binding curve of Braftide-Cy3 to the kinase domain of BRAF^{WT}. Fluorescence intensity was measured at a constant peptide concentration and varying protein concentrations on a Tecan fluorescence plate reader. The KD was obtained by fitting the data to a One-Site Binding equation in Origin. E) Coimmunoprecipitation of V5-tagged BRAF after pull-down of FLAG-tagged BRAF with or without braftide.

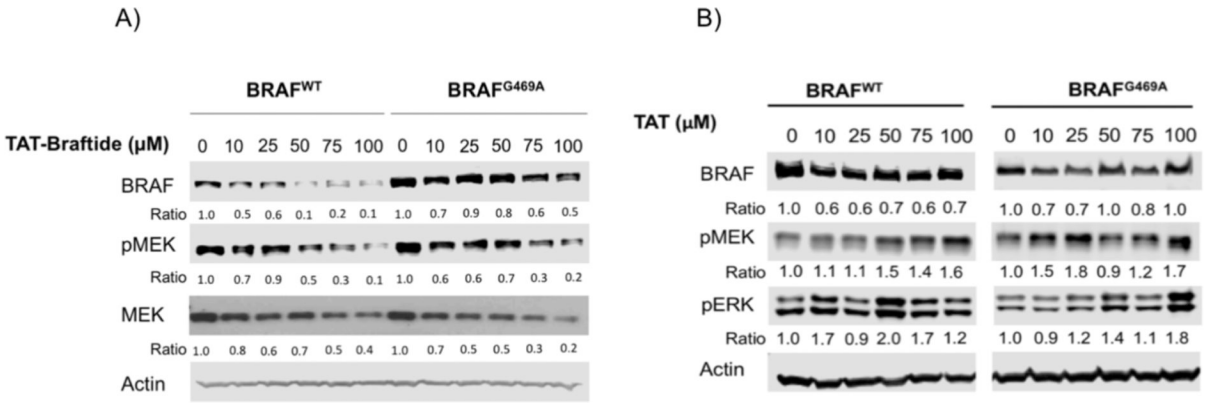

Figure 2. TAT-tagged Braftide inhibits BRAF$^{WT/G469A}$ kinase activity in HEK293 cells.

HEK293 cells transiently transfected with BRAF$^{WT/G469A}$ were treated with TAT-braftide or TAT peptide at the indicated concentrations (0, 10, 25, 50, 75, and 100 μM) for 4 hr. Cell lysates were subjected to immunoblotting with the indicated antibodies: anti-BRAF, anti-pMEK, and anti-Actin. A) Effect of TAT-braftide on BRAF$^{WT/G469A}$ kinase activity in HEK293 cells. B) Effect of TAT control peptide on BRAF$^{WT/G469A}$ kinase activity in HEK293 cells. Western blots are representative of at least three independent experiments. The band intensities from the Western Blots were quantified in ImageJ (NIH).

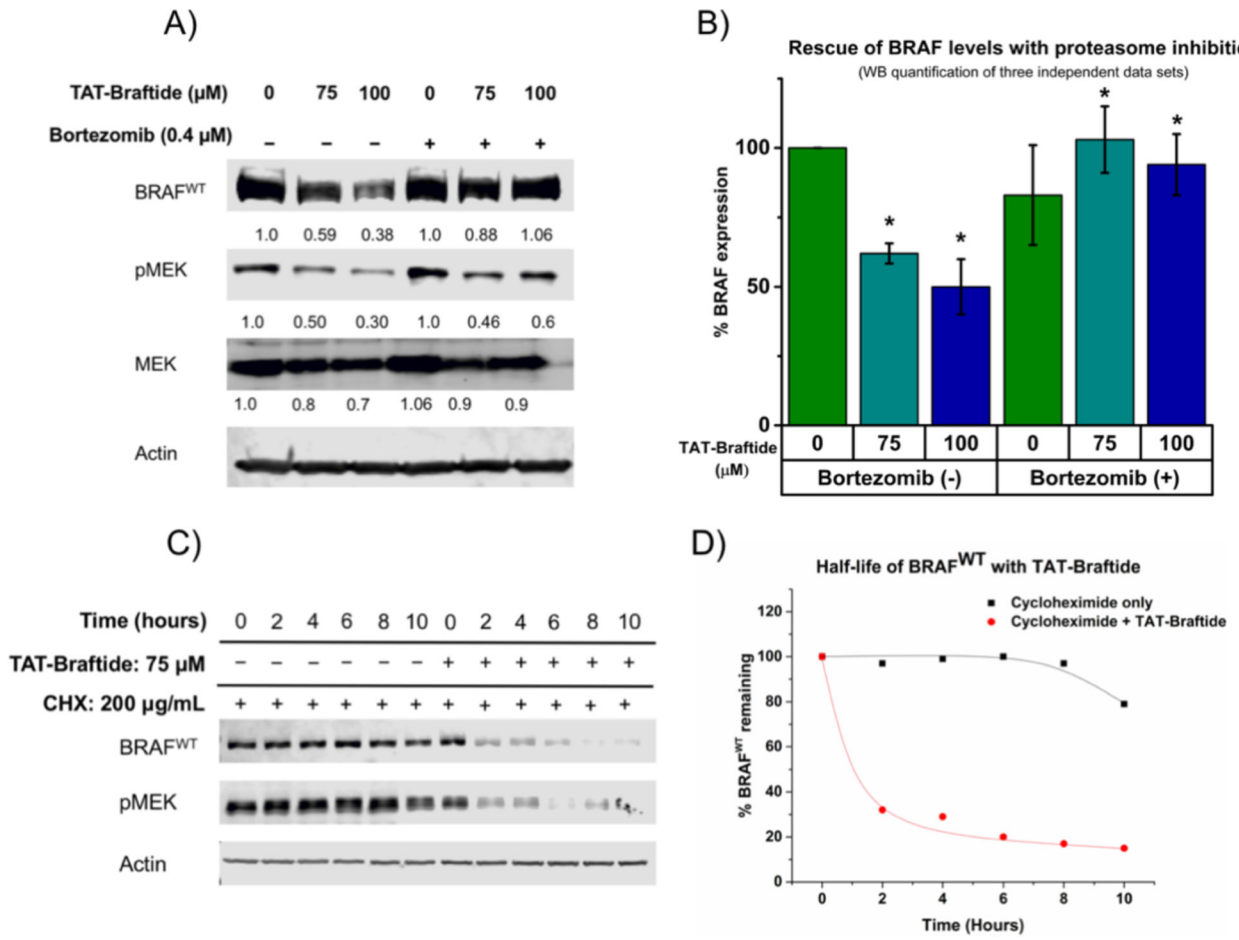

Figure 3. BRAF degradation with TAT-braftide treatment rescued through proteasome inhibition.

A) Proteasome inhibition with Bortezomib prior to TAT-braftide treatment rescued BRAF levels but not pMEK levels. HEK293 cells transiently transfected with $BRAF^{WT}$ were pre-treated with 0.4 μM of Bortezomib for 5 hr, followed by TAT-braftide treatment at the indicated concentrations (0, 75, and 100 μM) for 4 hr. B) Quantification of BRAF protein levels after rescue with Bortezomib. Band densities from three replicate Western Blots of (A) were quantified in ImageJ and then plotted in Origin. Results are expressed as mean ± SEM. (* p values < 0.01, from ANOVA) C) Half-life of $BRAF^{WT}$ with cycloheximide (CHX) treatment in the presence or absence of TAT-braftide. HEK293 cells transiently transfected with $BRAF^{WT}$ were treated with 200 μg mL^{-1} of cycloheximide alone or in combination with 75 μM of braftide for the indicated time points (0, 2, 4, 6, 8, and 10 hr). D) Quantification of the Western Blot in (C) to determine the half-life ($t_{1/2}$) of BRAF. Band densities from (C) were quantified in ImageJ and then plotted in Origin. Western blots are representative of at least three independent experiments.

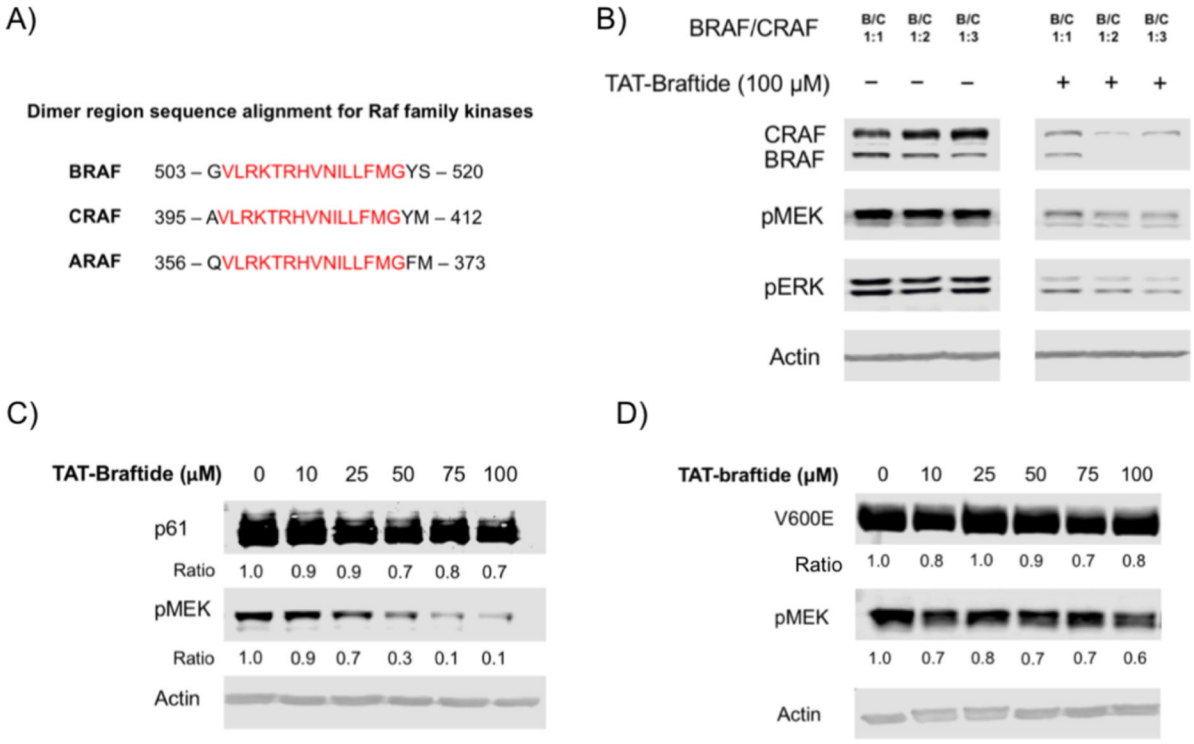

Figure 4. TAT-braftide inhibits the Kinase Activity of BRAF/CRAF Heterodimer in HEK293 Cells.

A) Sequence alignment of the dimer interface of three RAF family members, ARAF, BRAF, and CRAF. B) HEK293 cells transiently transfected with two plasmid constructs encoding Flag-BRAF and MBP-CRAF at three molar ratios, 1:1, 1:2, and 1:3. The cells were treated with 100 μM of TAT-braftide for 4 hr. C) HEK293 cells transfected with p61-BRAF^{V600E} were treated with TAT-braftide at the indicated concentrations for 4 hr. D) HEK293 cells transfected with BRAF^{V600E} were treated with TAT-braftide at the indicated concentrations for 4 hr. Western blots are representative of at least three independent experiments.

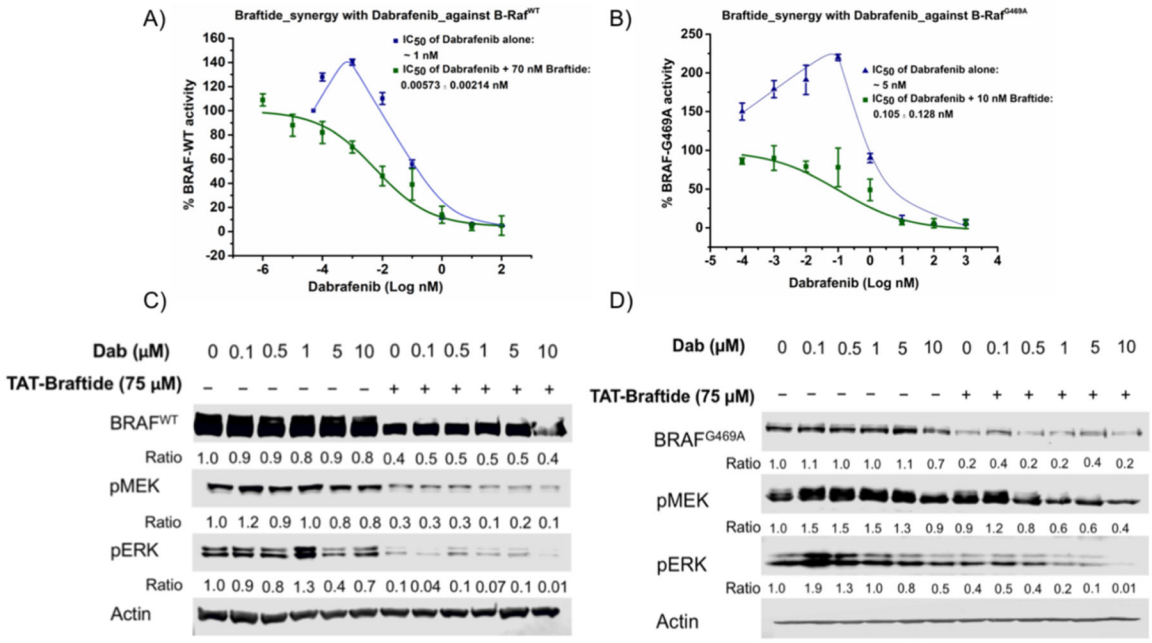

Figure 5. Combination treatment of braftide and dabrafenib abrogates paradoxical activation and improves dabrafenib efficacy.

A) Dose-response curves for braftide/ dabrafenib combination treatment and dabrafenib alone against FL-BRAF^{WT}. B) IC50 curves for braftide/ dabrafenib combination treatment and dabrafenib alone against FLBRAF^{G469A}. BRAF$^{WT/G469A}$ were pre-treated with braftide at concentrations lower than the IC50 (WT:70 nM; G469A: 10 nM) and then treated with dabrafenib at the indicated concentrations. IC50 values were obtained from a dose-response curve (4-parameter logistic equation) in Origin, from three independent experiments. C&D) Effects of combination treatment of TAT-braftide and dabrafenib in HEK293 cells overexpressing BRAF^{WT} or BRAF^{G469A}, respectively. Cells were pre-treated with TAT-braftide (75 μM) for 2 hr and then dabrafenib was added at the indicated concentrations (0. 0.1, 0.5, 1, 5, and 10 μM) for 1 hr. Western blots are representative of at least three independent experiments.

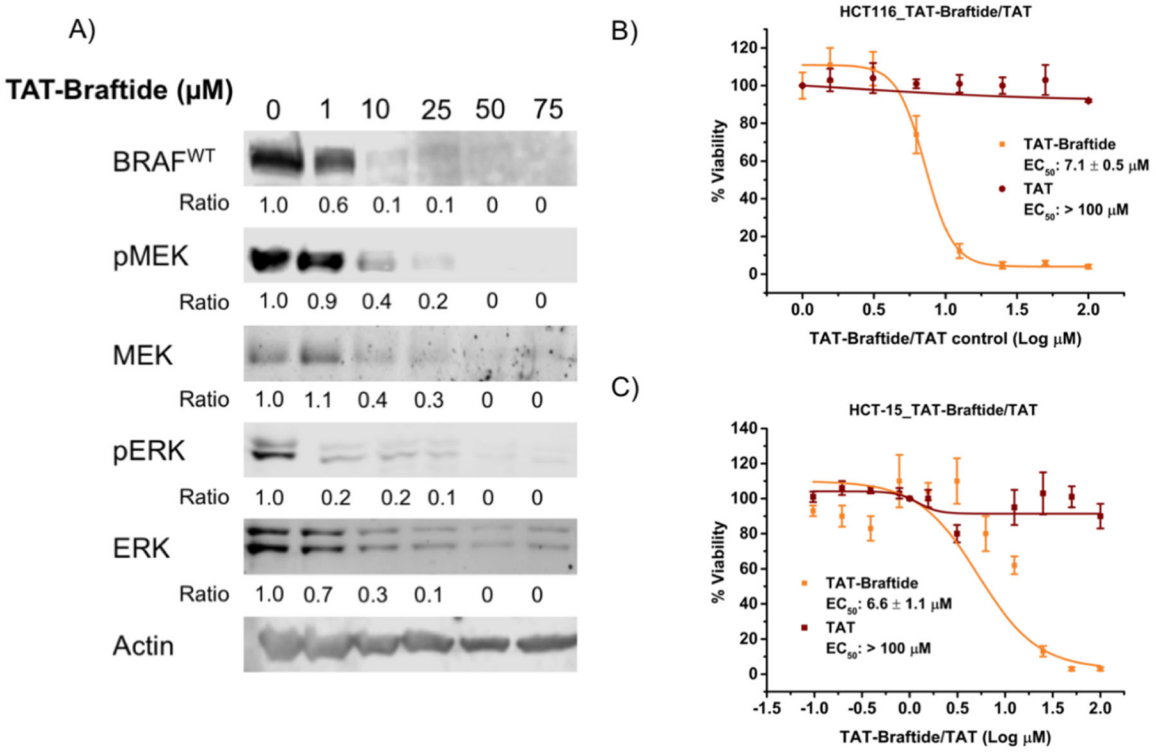

Figure 6. Braftide inhibits MAPK signaling and cell proliferation in KRAS mutated cell lines.

A) HCT116 cells were treated with braftide at the indicated concentrations (0, 1, 10, 25, 50, and 75 μM) for 4 hr.Western blots are representative of at least three independent experiments. Band densities were quantified in ImageJ. B&C) HCT116 (B) and HCT-15 (C) cells were treated with braftide or TAT control peptide at the indicated concentrations for 48 hr. Cell viability was determined through the WST assay according to manufacturer's instructions. IC50 values were obtained from a dose-response curve (4-parameter logistic equation) in Origin, from three independent experiments.

Table 1.

List of materials, reagents, antibodies used for experiments.

Reagent or Resource	Company	Catalog Number
Antibodies		
Mouse monoclonal anti-FLAG	Sigma	F1804–1MG
Mouse monoclonal anti-Actin	Sigma	A1978–200
Rabbit monoclonal anti-pMEK	Cell Signaling Technology	9154S
Rabbit monoclonal anti-pERK	Cell Signaling Technology	4370S
Mouse monoclonal anti-MEK	Cell Signaling Technology	4694S
Mouse monoclonal anti-ERK	Cell Signaling Technology	4696S
HRP-conjugated anti-rabbit	Cell Signaling Technology	70745
HRP-conjugated anti-mouse	Cell Signaling Technology	70765
IR-conjugated anti-rabbit	LI-COR	926–32211
IR-conjugated anti-mouse	LI-COR	926–68070
Cell Culture and Transient Transfections		
Dulbecco's Modified Eagle Medium	Gibco	11995–065
Phosphate-buffered saline	Gibco	10010–023
Fetal bovine serum	Gemini Bio-Products	100–602
L-glutamine	Gibco	25030–081
Trypsin-EDTA	Gibco	25300–054
Opti-MEM reduced serum media	Gibco	31985–070
Polyethyleneimine	Polysciences, Inc.	24765
Protein Quantification and Immunoblotting		
Bicinchoninic acid kit	Thermo Scientific	23225
Bovine serum albumin	Sigma-Aldrich	A7906–500G
Nitrocellulose membranes	Bio-Rad	1620115
Inhibitors		
Dabrafenib	SelleckChem	S2807
Vemurafenib	SelleckChem	S1267
Trametinib	SelleckChem	S2673
Cycloheximide	Sigma-Aldrich	C1988–1G
Bortezomib	SelleckChem	S1013
Peptides		
Braftide	Lifetein	Custom
Null-Braftide	Lifetein	Custom
TAT-PEGlinker-Braftide	Lifetein	Custom
TAT	Lifetein	Custom
Plasmids		
BRAF-WT-FL		
BRAF-G469A-FL		

Reagent or Resource	Company	Catalog Number
MEK-K97M		
ELISA Materials		
ELISA-glutathione-coated plates	Pierce-ThermoFisher	15420
SuperSignal™ ELISA Pico Chemiluminescent Substrate	ThermoFisher Scientific	37070