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Design and Synthesis of Type IV Inhibitors of BRAF Kinase that block dimerization and overcome paradoxical MEK/ERK activation

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Abstract

Despite the clinical success of BRAF inhibitors like vemurafenib in treating metastatic melanoma, resistance has emerged through “paradoxical MEK/ERK signaling” where transactivation of one protomer occurs as a result of drug inhibition of the other partner in the activated dimer. The importance of the dimerization interface in the signaling potential of wildtype BRAF in cells expressing oncogenic Ras has recently been demonstrated and proposed as a site of therapeutic intervention in targeting cancers resistant to ATP competitive drugs. Proof of concept for a structure-guided approach targeting the dimerization interface is described through the design and synthesis of macrocyclic peptides that bind with high affinity to BRAF and that block paradoxical signalling in malignant melanoma cells occurring through this drug target. The lead compounds identified are type IV kinase inhibitors and represent an ideal framework for conversion into next generation BRAF inhibitors through macrocyclic drug discovery.

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AUTHOR CONTRIBUTIONS

C.B. was responsible for the design, synthesis and purification of peptides.

Magda. R and G.K carried out the ITF and ITC assays.

M. R., S. B. and S.G carried out the cellular experiments

T.B. designed the cellular studies

C.M. was responsible for the project hypothesis, initial design of the linear and cyclic peptides and subsequent library design.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at DOI and includes plots for K_d determinations through ITF and ITC, an additional comparative table for peptide truncation data and HPLC and MS characterization of all the peptides synthesized in this study.

C.M. as well as an employee of the University of South Carolina is Founder, President and Chief Scientific Officer of PPI Pharmaceuticals, LLC however this company was not involved with this published study.

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INTRODUCTION

The Ras/Raf/MEK/ERK pathway involves the transduction of extracellular growth signals to the nucleus to regulate events in cell proliferation and differentiation. This pathway is frequently affected in tumor formation through the overexpression of growth factor receptors and activating mutations in Ras and Raf kinase are common events. Considerable efforts in drug discovery have been invested and have in recent years paid some dividends. In particular Raf kinases (ARAF, BRAF and Raf-1/C are known members) are considered as attractive therapeutic targets^{1, 2}. Of these, BRAF is the major activating kinase for MEK/ERK and as a result is probably the most frequently mutated kinase in cancers including melanoma, hairy cell leukemia and colorectal carcinomas among other tumor types^{3, 4}. A breakthrough in the treatment of malignant melanomas has been achieved in the approval of vemurafenib, a BRAF inhibitor initially producing dramatic responses in treated patients and which targets a constitutively active BRAF mutant (V600E). These drugs target the signal transduction pathways stimulated by binding of growth factors to their receptors that then result in activation of Ras proteins. Oncogenic Ras signaling occurs in about 30% of all human cancers and triggers homo- or hetero-dimerization of Raf-kinases that is critical for several aspects of signal propagation through downstream MEK and ERK kinases^{5, 6}. Despite intense efforts, pharmacologic inhibition of RAS proteins themselves and inhibition of their downstream effector kinases has so far been unsuccessful in treating RAS-driven tumors. Despite the dramatic initial response rates of vemurafenib in *BRAF* mutant melanoma patients, drug resistance and secondary neoplasms emerge in treated patients thereby dampening the initial enthusiasm for this approach^{7, 8}. Further investigation into the mechanisms driving these clinical complications has provided considerable insights and determined that a major cause results from “paradoxical MEK/ERK signaling” by the same mechanisms precluding the use of these drugs in Ras-driven tumors. These studies have demonstrated that while vemurafenib inhibits BRAF^{V600E} very potently, in the context of WT BRAF (in both homodimers and BRAF/C-Raf heterodimers) and activating Ras mutations, leads to kinase activation of the other partner in the dimer thereby stimulating the downstream pathway rather than inhibiting it^{9–11}. Allosteric transactivation of a catalytically competent RAF protomer by a drug-bound BRAF molecule requires an intact dimer interface¹². This resistance pathway therefore requires further efforts to complement inhibition of the mutant V600E kinase with other ways of inhibiting downstream signaling.

Despite clinical success, the emergence of resistant tumors necessitates continued investigation and drug discovery efforts around the Ras/Raf/MEK/ERK pathway. Combination of MEK inhibitors with approved BRAF drugs has been shown to be an effective strategy and has resulted in the recent approval of trametinib to treat BRAF mutated melanomas¹³. While a significant improvement, MEK inhibitors have some toxicity issues and thus further advances are required. ATP-competitive Raf inhibitors that induce paradoxical ERK activation must not be used to treat *RAS* mutant tumors^{12, 14}. A recent preclinical study has shown that targeting the complete Raf node phenocopies the growth inhibiting effects of removing the oncogenic driver, mutant Ras¹⁵. A new class of inhibitors that take the dynamic interplay of Raf-isoforms by dimerization and feedback loops into consideration would therefore be beneficial and this requires a detailed understanding of BRAF and its homo and heterodimerization and effects on downstream signaling. In this study, based on elucidation of the residues in the DIF important for dimer formation, the requirement for transactivation for an intact dimer interface^{12, 16} and published structural information on the BRAF dimer¹⁷, peptides were designed that successfully bind to BRAF and furthermore act to abrogate downstream signaling of ERK. These can be classified as type IV kinase inhibitors i.e. those that bind and inhibit allosterically at sites distant from the catalytic cleft¹⁸. The structure activity relationship of DIF peptides has been defined through computational analysis, alanine scanning and testing of these in an intrinsic tryptophan fluorescence (ITF) assay measuring direct binding to BRAF. Based on activities of the linear peptides and the observed loop structure at the dimer interface, highly potent cyclic peptides that mimic and stabilize the bioactive conformation have been generated. Macrocyclic drug discovery has in recent years become an area of interest especially in targeting protein-protein interactions^{19–22}. Macrocycles (MCs) typically go beyond the rule of 5 for orally available drugs especially with regard to allowing high MW compounds^{20, 23}. This enables more extensive coverage of the larger interfaces of PPIs. The lead BRAF DIF inhibitor macrocycles therefore represent tool compounds to probe how Raf dimerization events contribute to propagation of signals in the Ras/Raf/MEK/ERK pathway, while providing a scaffold for making drug-like cyclic peptides with favorable pharmacological properties.

RESULTS

Structure guided design and optimization of peptidic inhibitors of BRAF dimerization

The crystal structure of the homodimer of BRAF kinase (in complex with an ATP competitive inhibitor) has recently been solved²⁴. Furthermore, the dimerization of Raf kinases has been shown to involve a central cluster of residues known as the dimer interface (DIF). In particular, highly conserved basic residues within the DIF (R509 in BRAF) play a critical role in promoting dimerization²⁵ and DIF mutants incorporating the R509H mutation, either singly or in combination with the L515G and M517W substitutions, prevent mutant Ras and vemurafenib-induced paradoxical MEK/ERK signaling¹². Based on this information, the crystal structure of the BRAF homodimer (PDB ID: 4E26) was examined to determine if peptide sequences isolated from the DIF could potentially block the protein-protein interaction involved in BRAF homo and heterodimerization events. It was observed that the residues in the dimer interface occur in a looped turn structure (Figure 1) and

present a relatively compact binding interface with the other BRAF monomer (and presumably similar interactions in heterodimers with other RAF kinases).

Coincidentally, a study was published shortly after the initial peptide design, describing the cellular expression and effects on BRAF signalling of a 19-residue BRAF peptide, comprising residues 503–521 of BRAF¹⁶. To validate the approach of blocking the dimer interface through this sequence, DIF peptides were designed in cell permeable form as TAT fusions. These included TAT-pep1 (BRAF 503–521, prior study¹⁶), TAT-pep17 (BRAF 504–518, loop forming residues from DIF contact surface) and TAT-6alaNC3 (residues contacting the other monomer mutated to alanine). As shown (Supplementary Figure), results from a colony forming assay using EGF dependent MCF-10A cells as a preliminary screen for anti-proliferative activity demonstrated that TAT-pep1 completely inhibited growth at 2 μ M. The shorter BRAF sequence, TAT-pep17 had somewhat greater antiproliferative potency with complete inhibition at the slightly lower dose of 1.5 μ M and the negative control sequence had little effect on the colony forming ability of these cells (supplementary figure 1). Next, we tested the TAT-peptides on the human melanoma cell line Sbc12. This cell line does not carry BRAF mutations and is driven by the Q61K gain-of-function mutation in NRAS, an alternative and ERK pathway-activating event to BRAF mutations in melanoma¹². We first asked whether the melanoma cells efficiently take up the TAT peptides. To this end, we used TAT peptides with an N-terminal 5-Carboxyfluorescein (5-FAM) label. As shown in Figure 2A, melanoma cultures exposed to 3.6 μ M of both peptides accumulate fluorescently labelled peptides, while cells exposed to the lower concentration only display a diffuse autofluorescence. Interestingly, the cells treated with the biologically active FAM-TAT-Pep17 not only form smaller colonies, but also tend to display a round morphology, while control peptide exposed cells attached to the substrate are more elongated. The dose dependent effect of TAT-Pep17 on proliferation was also evident in a colony formation assay (Figure 2B). Similar results were obtained for the unlabelled versions of the TAT-peptides (Supplementary Figure 2). In summary, these data show that both labelled and unlabelled TAT-peptides matching to the BRAF DIF impair proliferation, while the control TAT-6alaNC3 peptides only show effects at higher concentrations.

Having achieved cellular validation for on target inhibitory activity of BRAF dimerization, an intrinsic tryptophan fluorescence (ITF) assay was developed to quantify binding of these peptides to BRAF. A tryptophan residue (Trp450) directly contacts R509 thereby providing the impetus to use this assay format to quantify binding. Since there is no competitor involved in this assay, the readout is the K_d for direct interaction between the peptide and the BRAF construct (Tables 1, 2, and 3, see supplementary Figure 1 for an example K_d curve). For this assay, a truncated BRAF comprised of the kinase domain in addition to the dimerization interface was expressed and purified. Peptide 1 comprising BRAF residues 503–521 and shown above to inhibit cellular proliferation as a TAT fusion, was found to have a K_d of 3.8 μ M thus confirming affinity for the BRAF monomer¹⁶. Two negative control peptides based on mutation of residues shown to be important for dimer interface in the endogenously expressed BRAF mutants¹² were tested. As expected, the triple mutant (2; R509H, L515G, M517W) had no detectable binding. Following confirmation of the binding of the initial peptide and lack of affinity of the negative control, a peptide library (Table 1) was designed around the initial sequence and synthesised using solid phase peptide

synthesis. Computationally designed mutations were incorporated to probe roles of specific residues in binding to BRAF and affecting dimer formation. The aim of this library was to probe the binding determinants and effects of truncation with the eventual goal of generating cyclic peptides that stabilize the loop structure observed in the dimeric crystal structure.

A scrambled peptide (**3**, 2.96 μM) was previously reported as a negative control with no effect on BRAF functional activity¹⁶. In contrast, however, when tested in the ITF assay, was shown to have similar and even slightly improved binding compared to the native BRAF sequence. Alignment of its sequence with BRAF shows that the key DIF interface residues are preserved in this peptide. Peptide **4** revealed that L505 plays little role in binding to the DIF as evidenced by an equipotent K_d of the alanine replacement peptide (compared to **1**). From the crystal structure (4E26) it was observed that the first arginine of the sequence (R506) potentially disfavors binding due its role in making an adjacent salt bridge less energetically favorable. Synthesis and testing of the R506E mutant confirmed this hypothesis as shown by the lowered K_d value (**5**, 1.09 μM). Removing the charge in the R506L mutant was even more beneficial for inhibiting the dimer in the doubling of activity and 8-fold (**6**, 0.54 μM) relative to the native BRAF sequence. Replacement of T507 with both Asp (**7**) and Ala (**8**) resulted in a marginal increase in binding as indicated by the slightly reduced K_d values of these peptides. H510F had a complete loss of affinity.

Two hydrophobic residues (**10**, V511; **11**, L514) were also shown to contribute to the affinity of the WT BRAF as evidenced by decreased binding of the alanine mutants, but more so in the L514A context. The conservative mutations at L515 included Ile and homoleucine and these were shown to have minimal effect and a significant increase in potency respectively (**12**, L515I, 4.1 μM ; **13**, L515homoLeu, 1.25 μM). Computationally, the F516D mutation (**14**) was predicted to increase binding however the opposite effect was observed with no binding being detected using the intrinsic tryptophan fluorescence assay.

Truncation of the C-terminal tripeptide, YST to generate BRAF 503–518 resulted in a 2-fold increase in binding affinity (**15**, 1.88 μM) relative to BRAF 503–521. Further truncation of the N-terminal glycine from this molecule led to a more dramatic increase of almost 30-fold (Table 2; **17**, 0.13 μM). Replacement of the C-terminal carboxylate with an amide group (**18**, 0.48 μM) resulted in a significant loss of affinity (3–4-fold). Furthermore, acetylation of the N-terminal amine (**19**, 0.8 μM) led to an even greater drop-off in binding affinity. With the observed 30-fold decrease in K_d for N and C-terminally truncated peptide (**17**, 504–518) relative to the initial 503–521 sequence tested (**1**, 3.84 μM), it was determined that this was an ideal scaffold to further investigate the contributions of each residue to binding through alanine scanning mutagenesis (Table 2). Results from testing of L505A (**21**, 0.45 μM), R506A (**22**, 0.36 μM), F516A (**29**, 0.57 μM), M517A (**30**, 0.54 μM), revealed that these positions are relatively insensitive to substitution compared to others including R509A (**24**, 2.4 μM), H510A (**25**, 2.7 μM), N512A (**26**, no binding) and I513A (**27**, 2.7 μM) which underwent a more than 10-fold potency drop-off. L514A (**30**, 1.02 μM) had a significant but less drastic decrease in affinity. After completion of the alanine scan, peptides were synthesized performing additional truncations from both the N and C-terminal ends. Truncation of the N-terminal valine producing BRAF 505–518 resulted in essentially equipotent binding affinity (**31**, 0.19 μM) relative to **17** while removal of the C-terminal

glycine from **15** had a much more consequential effect and led to a steep drop off in activity (**16**, 5.75 μM). Relative to **1** however, it can be seen that truncation of the initial DIF peptide from both N and C-terminus by 1 and 4 residues respectively led to compromised binding (**16**, 5.75 μM). These studies suggest that BRAF residues 505–518 are the minimal recognition motif for potent dimer breaking activity.

Design of cyclized BRAF peptides with enhanced binding affinity.—From the crystal structure of the BRAF dimer, it was observed that residues 505–516 are in close proximity and that the sequence between these residues forms a loop without the side chains of the termini contributing to the protein-protein interaction (Figure 1). This observation suggested that a cyclic peptide could be generated to rigidify the peptide, stabilize this loop conformation and thereby decrease the entropic cost of binding in turn generating more potent inhibitors relative to the linear sequences. Recently there has been considerable attention given to the development of macrocyclic compounds as potential drug leads and where cyclization has been shown to increase potency, impart cell permeability and furthermore improve metabolic stability¹⁹.

To obtain proof of concept for macrocyclic BRAF inhibitors, the disulfide cyclized molecule was synthesized incorporating cysteine residues replacing L505 and F516 which are close in the crystal structure and whose side chains when replaced with alanine resulted in diminution but not total loss of activity. Testing of this cyclic peptide showed that it underwent a 15-fold increase in binding (Table 3, **33**, 0.36 μM) relative to similar linear sequence (**16**) therefore confirming hypothesis for constraining the peptide sequence. In addition to these points of cyclization (504–518, Figure 4C), another potential connection point for a covalent restraint is between the side chains of position 508 and 513 which occur as Thr and Ile respectively in the native sequence. Analysis of the crystal structure suggests that these residues also are in close proximity and do not contribute directly to binding. An orthogonal cyclization strategy was designed for introduction of a lactam bridge between these residues through use of highly acid sensitive side chain protection. Synthesis of cyclo 508–513 T508O, I513E revealed that substantial activity was observed for the cyclic molecule (**35**, 0.78 μM) whereas no detectable binding was obtained for its linear counterpart (**34**) which contained the same mutations but not in a lactam bridge. Interestingly substitution of N512 for alanine resulted in a potency increase for the non-native substitution (**36**, 0.46 μM). Expansion of the lactam bridge by one methylene through use of lysine instead of ornithine in the orthogonal protection strategy resulted in a potency decrease in the N512 context (**37**, 1.89 μM) but a substantial increase in the N512A cyclic version (**38**, 0.061 μM).

Isothermal Titration Calorimetry (ITC) of BRAF binding of DIF Peptides.—As confirmation of binding affinity in an alternate format and to investigate the thermodynamics of binding for DIF peptides to BRAF, ITC experiments were carried out for three peptides shown to interact with BRAF through ITF measurements (See supplementary Figure 2 for a K_d curve generated by ITC). Peptide **8** (ITF K_d = 2.8 μM) was shown to be somewhat less potent by ITC however the K_d result was variable (K_d = 14.9 $\pm$ 10.8 μM ; ΔH = –34.8 kJ/mol; ΔS = –28.4 J/mol·K). Peptide **17** (ITF K_d = 0.13 μM) had a comparable affinity as

measure by ITC with a K_d found to be $0.35 \pm 0.17 \mu\text{M}$. As expected for a flexible linear peptide (also for **8**) with high entropy in the free state, the binding is primarily enthalpy driven ($\Delta H = -199 \text{ kJ/mol}$) and the entropy term is unfavorable for the overall free energy ($\Delta S = -567 \text{ J/mol}\cdot\text{K}$). The cyclic derivative, **36** also had a comparable K_d as measured by ITC ($K_d = 0.31 \pm 0.16 \mu\text{M}$) to that obtained with the ITF assay ($K_d = 0.46 \mu\text{M}$). As expected and in line with the rationale for generating cyclic peptides, binding of the macrocyclic peptide is driven more by entropic factors than enthalpic ($\Delta H = -9.41 \text{ kJ/mol}$ and $\Delta S = 92.05 \text{ J/mol}\cdot\text{K}$).

DIF Peptides block paradoxical activation of BRAF induced by vemurafenib.—

As described above, a major driving force for the development of DIF inhibitors is to provide an alternate therapeutic strategy to overcome the observed resistance to approved BRAF inhibitors. Drugs such as vemurafenib cause “paradoxical activation” of the MEK/ERK pathway in RAS mutant cells, thereby contributing to primary and acquired BRAF inhibitor resistance^{10, 11, 26, 27}. Since this resistance mechanism requires an intact dimer interface¹², the ability of DIF peptides (Peptide **1**, Table 1 and FAM-TAT-Peptide17) were tested for their ability to overcome signaling downstream of BRAF induced by the ATP competitive inhibitor vemurafenib (PLX 4032). As shown previously for NRASQ61K driven human Sbc12 melanoma cells in the absence of DIF targeting peptides^{12, 28}, BRAF inhibition induces paradoxical MEK/ERK pathway activation in control peptide treated Sbc12 cells, as reflected by increased levels of phosphorylated MEK (pMEK) and ERK (pERK). When introduced through electroporation, **1** effected a dose dependent inhibition of paradoxical MEK/ERK activation induced by this drug in a *NRAS*^{Q61K} mutant melanoma cells. Due to the inefficiencies of electroporation, relatively high concentrations were required however almost complete inhibition of vemurafenib induced phosphorylation of MEK and ERK was observed at $300 \mu\text{M}$ peptide. To test the effects of the TAT-DIF peptide, shown to strongly inhibit the growth of the melanoma cell line (Figure 1), and to transfect DIF inhibitory peptides in a more gentle manner, Sbc12 cells were treated with FAM-TAT-Pep17 and FAM-TAT-Pep6alaNC3. Interestingly, this paradoxical signalling effect was absent in FAM-TAT-Pep17 treated cells, and these also showed a reduction in RAF-1 levels. This is of particular interest as RAF-1 plays an important role as a MEK activator in NRAS driven melanoma, (including the Sbc12 model)²⁹, and represents an important dimerization partner for BRAF in models of paradoxical ERK pathway activation by either drug-bound or kinase-dead BRAF molecules^{12, 30}. Furthermore, to monitor an important biological endpoint of MEK/ERK signaling in melanoma cells, we assessed the expression and phosphorylation of FRA1, the product of the immediate early gene *FOSL1*. *FOSL1* has a well-documented role as an ERK target gene and phosphorylation of its product, FRA1 at S265 (pFRA1), serves as an excellent read-out for long-term persistence effects of ERK activity³¹. As shown in Figure 3, vemurafenib induced FRA1 expression and phosphorylation in control cells but not in FAM-TAT-Pep17 treated cells. In summary these data conclusively show that introduction of peptides into Sbc12 melanoma cells quenches vemurafenib induced paradoxical ERK pathway activity.

DISCUSSION

The results from the design and K_d evaluation of BRAF DIF peptide inhibitors, demonstration of their antiproliferative activity and subsequent proof of concept in their ability to block downstream activation of MEK/ERK promoted by vemurafenib, confirms the potential of this strategy for next generation BRAF inhibitors. By monitoring the expression and phosphorylation of the transcriptional target and protein substrate of ERK, FRA1, the observation has been made that DIF peptide inhibitors impair a critical node in melanoma pathobiology, since FRA1 plays an important role in NRAS and BRAF driven melanoma^{32,33}. Targeting the dimer interface through such type IV kinase inhibitors should also be an effective strategy against non-V600E BRAF point mutants or BRAF fusion proteins that require an intact dimer interface and are intrinsically vemurafenib resistant as a result of their increased homodimerization and/or mutations potentially interfering with drug uptake^{35, 36}. Indeed, genetic approaches using the R509H mutation revealed an essential role for the paradoxical activity of the kinase-dead BRAF^{D594A} mutant (PMID 22510884). BRAF mutations affecting D594, a residue essential for catalysis, represent the third most common class of BRAF mutants in cancer (PMID 20141835) and these and other kinase-inactivating or impairing mutations even outnumber the canonical V600E substitution in tumor entities such as non-small cell lung cancer (PMIDs 30653256, 29540830). Likewise, the R509H mutation impairs the transforming potential of intermediate activity BRAF mutants found in cancer and RASopathy patients such as Q257R and F595L (PMID: 22510884) and oncogenic BRAF fusion proteins (PMID 23533272), which are frequently encountered in human cancer due to advances in sequencing technologies (PMID 29540830). Insensitivity to ATP competitive inhibitors makes treatment of these tumors problematic and therefore new modalities such a dimerization inhibitors would provide new therapeutic options.

Having demonstrated in cellular studies the importance and relevance of the DIF for RAF kinase drug discovery, the detailed structure-activity relationship information obtained from testing of the peptide libraries provides key insights into the minimal recognition motif and the binding determinants of dimer blocking. This information will be of profound importance in the optimization of binding affinity, metabolic stability and drug-like properties of the peptidic inhibitors in both the linear and cyclic contexts and lead to a drug-like macrocyclic RAF kinase dimer breaker. Furthermore, the SAR data can be interpreted in structural terms through examination of the protein-protein interactions present in the dimeric BRAF (Figure 4A-C, PDB ID 4E26) and the extrapolation of these to the peptide-protein context. In the following discussion, residues of the BRAF peptide are given as one letter amino acid codes while those of the BRAF protein are described by 3 letter residue codes. Firstly, in the BRAF 504–518 context, the L505A mutation leads to a potency decrease not through loss of binding contacts but more likely through disruption of a stabilizing intramolecular interactions with F516 explaining the almost 4-fold potency loss of peptide **21** relative to **17**. R506 when mutated to alanine resulted in a 3-fold loss of potency and which can be rationalized by absence of contacts to Asp448 (Figure 4C). The alanine side chain however has additional interactions with F516 offsetting the loss of the ion pairing interaction. The K507A peptide proved to be highly insoluble and therefore

could not be tested. In the crystal structures, K507 is adjacent to Asp448 however does not appear to have a close ionic interaction and therefore its replacement should have minimal impact. T508 was demonstrated in the larger peptide context to be relatively insensitive to substitution in that its replacement with both Asp and Ala led to a mild increase in binding affinity. Both the T508 backbone amide NH and its side chain hydroxyl makes a bifurcated H-bond to the backbone carbonyl of L505 and therefore the loss of the side chain H-bond may result in an increase in strength of the backbone-backbone interaction.

As expected and validating the mutagenesis studies with the full-length BRAF¹², R509 in the peptide inhibitor context is very sensitive to mutation since R509A has an 18-fold loss of activity. R509 has an unusual binding motif since it is buried and forms an antiparallel interaction with the corresponding residue in the other monomer of the dimeric structure (Figure 4B and C). The charge-charge repulsion of this motif is probably offset by side chain ionic interactions with the helix dipole of the other monomer. If the charges are stabilized on the two guanidine nitrogens (since a guanidinium group has three resonance structures; one from each monomer or from the peptide-monomer complex) that interact with the helix dipole, then the repulsive forces between the two arginine residues would be minimized due to the distance. R509 also interacts with Trp450 of BRAF through a pi-cation interaction (Figure 4C) further illustrating the contributions of this residue.

H510 makes an extensive network of intermolecular H-bonds (with the backbone carbonyl of His447 of the other BRAF monomer) and intramolecular hydrogen bonds (with N512 sidechain stabilizing a reverse turn) and therefore explaining its similar potency loss to R509 when mutated to alanine. The complete loss of binding for the N512A substitution is harder to rationalize in structural terms since it only has intramolecular contacts as opposed to H510 which also has intermolecular interactions. BRAF residues 510–513 (HVNI) make up a type II β -turn and therefore it is likely that the alanine substitution has more of a conformational effect. Statistical analyses of β -turns indicate that asparagine is among the most frequently occurring residues at the i+2 position (His is the i position) and alanine is one of the least probable³⁴. It is thus likely that in the linear context, the His-Ala substitution is quite destabilizing for the turn structure and in turn has a significant impact on the presentation of the binding determinants and entropy of binding. I513 (the i+3 residue in the turn) has few protein contacts but possesses important sidechain-sidechain interactions to both T508 and H510. The loss of these interactions in the alanine replacement peptide and the significant loss in binding affinity underscore the importance of the β -turn conformation to efficient binding to BRAF in the linear peptides.

From the crystal structure, it appears that L514 makes neither intra nor intermolecular contacts in line with a more moderate potency decrease after modification to alanine. Mutation probably results in increased rotational entropy of the alanine containing peptide disfavoring nucleation of the β -turn structure. The importance of L515 to binding is underscored by an extensive network of interactions with the lipophilic portion of the side chain of Arg509 and with the backbone of His510. Arg509 of the BRAF monomer receptor forms a bridge over the pocket that the cognate R509 residue from the peptide inserts into. L515 also has moderate Van der Waals contacts with M517 thereby providing intramolecular conformational stabilization. Further to this, synthesis and testing of F516A suggests a less

involved role of the aromatic side chain in binding to BRAF. The 3-fold drop in affinity was consistent with the interactions of the sidechain both intramolecularly (as mentioned above with L505 and R506) and intermolecularly with Arg509 of the monomer. M517 alanine substitution led to a loss comparable to that induced by F516 mutation. This was explained by numerous contacts to Arg509 of the monomer and to His510 of the BRAF protein.

As described above, truncation studies were included in the peptide library design since optimization of molecular weight is a key parameter defining drug-likeness and oral bioavailability in beyond rule of 5 (bRo5) space^{20, 23} (For a direct comparison of the peptides in the truncation series see Supplementary Table 1). Truncation of BRAF 503–521 from both the N and C-termini resulted in an optimized sequence (**17**, 504–518) with a sub-micromolar K_d. The 30-fold potency enhancement can be surmised from the BRAF dimer crystal structure and likely results from optimal positioning of the C-terminal carboxylate (i.e. when G518 is the C-terminal residue) for interaction with the R506 sidechain to form a putative cyclic structure stabilized by an ion pairing interaction. Removal of the charge by replacement with a C-terminal amide (**18**) and the resulting decrease in binding provides additional evidence for this. The 10-fold lower affinity of the Gly N-terminal extension (G503, **15** compared with **17**) is harder to explain in structural terms however it appears that in this sequence the free amine of the N-terminus (positively charged) is positioned to compete with R506 for binding to Asp448. This alternative binding mode may in turn promote conformations that are sub-optimal in terms of interactions of the rest of the peptide with BRAF. The peptide with the N-terminal valine (**31**) removed had similar potency to **16** further confirming that this residue does not in itself contribute significantly to binding but that the side chain to head salt bridge interaction promotes a favorable bound conformation.

As described above in the initial rationale for the project, the existence of a loop structure in the dimer interface provided the impetus for stabilization of a potential peptidic inhibitor through a covalent restraint. Due to the β -turn structure, the BRAF sequence 505–516 loops back on itself with Leu505 and Phe516 being close to each other in space. The observation that these residues do not contribute significant intermolecular interactions makes them ideal candidates to generate a cyclic peptide. Peptide **33** has a disulfide bridge replacing these two residues which led to a 16-fold increase in affinity as measured by the K_d value compared to **16**, the native BRAF linear counterpart. This confirms that macrocyclic peptides stabilizing the loop conformation provide a beneficial contribution to the binding free energy and this occurs putatively through decrease in the entropic cost of binding. With disordered linear peptides, their $T \Delta S$ contribution to binding is positive when the molecule assumes a more rigid bound conformation ($\Delta S = S_{\text{bound}} - S_{\text{free}}$) thereby overall making ΔG more negative. Cyclization generally lowers the S_{free} value by paying the entropic penalty during the cyclization reaction. Further to the 505–516 cyclized molecule, another cyclic variant was generated by incorporating a lactam (cyclic amide) bridge between residues 508 and 513 which also are close in space in the crystal structure. This would generate a smaller 6 residue cyclic peptide compared to the larger 12 residue 505–516 macrocycle. While T508 makes minimal contributions to binding, I513 substitution led to a 20-fold decrease in affinity due to the loss of intramolecular contacts. Decreased interactions in replacing the hydrophobic side chain with glutamate should be compensated however through the

formation of the covalent restraint since this would make the intramolecular contacts redundant. The results obtained for the six residue (and with the 12mer) macrocycles fully validate the initial design rationale in generating cyclic molecules in that highly potent ligands for BRAF have been obtained and thus have the potential for further development as next generation therapeutics.

The data obtained for testing the 4 cyclic 508–513 variants provides insights into the conformational determinants required for optimal binding to BRAF and for macrocyclic drug discovery of this cancer target. It is apparent from the K_d results (Table 3) that the ornithine derivatives are binding in a suboptimal conformation since they are less potent than the linear derivatives (peptides **35**, **36**). The extra methylene in the lysine to glutamate cyclized molecule (**38**, Figure 4A) provides the necessary flexibility to promote more favorable non-bonded interactions with BRAF. It is therefore the best framework for optimization and occurs when the native asparagine residue at position 512 is exchanged for an alanine. The K_d of 61nM for Peptide **38** (K508, N512A, E513) provides strong conformation for the macrocycle approach in that a cyclic DIF inhibitor was the most potent compound identified. In contrast, in the native context (N512), the lysine cyclized molecule **37** (K508, E513) was a weaker binder than either of **35** (O508, E513) and **36** (O508, N512A, E513), a somewhat anomalous result. A possible explanation is that residue 512, as discussed above, is likely crucial to stabilization of the β -turn (HVNI), therefore should have a significant effect on the overall cyclic conformation and how tightly the molecule binds. Since the N512A exchange in the linear context led to loss of binding, computational analysis was performed for the cyclic peptides and suggested that the alanine substitution in the peptide **38** context allows more flexibility for the turn structure which may be necessary for optimal binding given the covalent bridge between residues 508 and 513 (Figure 4A).

The results obtained from the ITF assay were validated using isothermal titration calorimetry and confirmed that the binding affinities for three peptides compared favorably between the two methods. ITC data also corroborated the macrocyclic design hypothesis in that the linear peptides had favorable enthalpy of binding and the cyclic version had beneficial entropic contributions to ΔG as expected. Results for **35** were in line with the observation that the reduced affinity for the ornithine cyclized version is due to an unfavorable conformation, leading to suboptimal interactions with the BRAF pocket and with the result that adding an extra methylene group to the bridge alleviates this leading to significantly increased binding affinity.

CONCLUSIONS

The preliminary validation for the approach of targeting the dimerization interface of BRAF (and heterodimers with other RAF kinases) through peptide inhibitors has been successful. Despite the clinical success of BRAF inhibitors like vemurafenib in treating metastatic melanoma, resistance has emerged through “paradoxical MEK/ERK signaling” where transactivation of one protomer occurs as a result of drug inhibition of the other partner in the activated dimer. Through a structure-guided approach, linear and macrocyclic peptides targeting the dimerization interface have been identified and shown to bind with high affinity to BRAF. The lead cyclic molecule is a type IV kinase inhibitor and represents a promising

scaffold for macrocyclic drug discovery where further ring stabilization, truncation, N-methylation and incorporation of non-natural amino acids have been shown to improve drug-likeness and pharmacological properties. Furthermore, DIF peptides efficiently exhibit anti-proliferative activity and inhibit paradoxical signalling in malignant melanoma cells stimulated by vemurafenib as evidenced by decreased levels of phosphoMEK/ERK and downregulation of the ERK target gene *FOSL1*. Targeting the dimer interface through type IV BRAF kinase inhibitors provides a new strategy to target non-V600E BRAF point mutants or BRAF fusion proteins since these require an intact dimer interface, are vemurafenib resistant and therefore pose a significant challenge. Overall DIF inhibitors show considerable promise for further development as next generation RAF kinase inhibitors as anti-tumor therapeutics.

EXPERIMENTAL SECTION

Solid Phase Synthesis of Linear Peptides:

The synthesis of all peptide analogs was accomplished using standard Fmoc chemistry. The linear sequences were synthesized on H-Rink Amide ChemMatrix resin using a Protein Technologies Prelude peptide synthesizer. Initially, the resin was swelled in DMF 3 times followed by 5 min washes. Amino acid coupling reactions were accomplished with Fmoc protected amino acids (3 eq), HATU (4 eq), and DIPEA (8 eq), the reagents were dissolved in DMF (5 mL) and the reaction was mixed via nitrogen bubbling for 2 hours at room temperature. Following coupling, the reaction vessel was drained and the resin is washed 3x with DMF, 3x with DCM, and 3x with DMF again. For Fmoc deprotection, the resin is treated with a solution of piperidine (20% in DMF) 2× 10 minutes. Again, the resin is washed as previously stated and the process is repeated for each respective residue in the defined sequence. The sequences of the TAT-labeled peptides (Figures 2 and 3) are as follows: TAT-Pep1, GRKKRRQRRR (PEG2) GVLKRTRHVNILLFMGYST; TAT-PEP17, GRKKRRQRRR (PEG2) VLRKTRHVNILLFMG, TAT-6ALANC3, GRKKRRQRRR (PEG2) GVLAATAAVNALLFAGYST, FAM-TAT-PEP17 and FAM-TAT-PEP6ALANC3 are labelled at the N-terminus with 5-FAM as a fluorescent tag.

Peptide Cyclization Reactions:

Side chain-to-side cyclization was accomplished by one of two methods, either through a lactam linkage or through a disulfide bond linkage. Cyclization residues with orthogonal protecting groups were chosen to be able to selectively deprotect the side chains of specific residues without affecting the rest of the peptide. For the lactam method, the amine residue's side chain was protected with Mtt and the acid residue's side chain was protected with 2-O-PhiPr. Both of these protecting groups can easily be removed by treatment (7× 3 min) of the resin with a low concentration of TFA (2%) in DCM. Once the orthogonal protecting groups have been removed, overnight treatment with HATU (4 eq) and DIPEA (8 eq) was used to effectively cyclize the linear, partially deprotected peptide. For the disulfide cyclized peptides, the cysteine residues involved in the cyclization were orthogonally protected with Mmt, which can easily be removed by treatment (7× 3 min) of the resin with a low concentration of TFA (2%) in DCM. Following deprotection, the two cysteine side chains can be oxidized to form the disulfide bridge by treatment with a solution of NCS (2 eq)

dissolved in DMF for 15 minutes at room temperature. Following cyclization, peptides are cleaved from the resin by treatment with a solution of TFA/TIPS/H₂O (94/5/1) for two hours. The cleavage solution was drained from the synthesis vessel and the solvent evaporated to yield the crude product.

Purification of Cyclic Peptides:

The crude peptide product was precipitated several times from cold ethyl ether and filtered through a fritted funnel to remove the majority of the scavenged protecting groups. The precipitate is then dissolved in a solution of ACN/MeOH/H₂O (1:1:1) and purified by 500 µL injections onto a Phenomenex C18 semi-preparative column until a purity of 95% is reached. Separation is accomplished using a standard water/acetonitrile (0.1% formic acid) mobile phase with a separation gradient of 5–45% B over 40 minutes and purity was confirmed using a 4.6 × 250 mm analytical column and a gradient of 5–95% acetonitrile/water/0.1%FA/30 min. Fractions are characterized via mass spectrometry, combined, and purity is evaluated by injection on the analytical LCMS column (For full characterization data for all peptides included in this study see Supplementary Table 2).

Dissociation Constant (K_d) Determination from ITF and ITC Measurements

The dissociation constant is an indicator of binding strength between two molecules. For the reaction: P + L ↔ PL

K_d is expressed by the equation: $K_d = \frac{[P][L]}{[PL]}$

where [P] is the concentration of free Protein, [L] is the concentration of free Ligand and [PL] is the Ligand-bound-Protein.

Fluorescence intensity was measured with a Hitachi F-2500 fluorescence spectrophotometer. Briefly, 1.6 mL of protein solution (0.5 µM) was placed in a cuvette and equilibrated at 15 °C for 1 h. After equilibration, small increments (2–15 µL) of the ligand solution were injected in the cuvette. The ITF and ITC experiments were performed in 20 mM Hepes buffer (pH 7.5), 10 mM MgCl₂, 30 mM NaCl. For certain ligands that were insoluble in aqueous media 5–10% DMSO was added to increase its solubility). The slits were set at 10 and 10 nm in the excitation and emission respectively. In order to determine dilution effect of BRAF (due to ligand addition) and any fluorescence effect by unbound ligand, a blank sample containing Trp with the same fluorescence signal, was titrated with ligand additions as described above. The sample absorbance was kept below 0.1 to minimize the inner filter effect³⁷.

The K_d of BRAF/ligand was calculated by fitting fluorescence data using the one-site binding site model in Origin 7 as follows:

$$[L_{total}] = \frac{2 \cdot \theta \cdot [P_{total}]}{K_b \cdot (-K_{diss} + \sqrt{K_{diss}^2 - 4 \cdot K_{diss} \cdot [P_{total}] \cdot (\theta - 1)})} + \theta \cdot [P_{total}] \quad \text{Eq. (1)}$$

ITC was measured with an Affinity ITC instrument (190 μ L cell volume, TA Instruments, USA) at 15 °C with stirring speed 170 rpm. The sample cell was loaded with the solution of 6.5–10 μ M of protein and the 50–1000 μ M peptide inhibitor solution was placed in the injection syringe. In a typical experiment, 12 injections of 2 μ L aliquots of the peptide were added into the calorimeter cell. Data analysis was performed using NanoAnalyze software according to model of the single set of identical independent sites. Also two “blank” experiments was performed with the above settings.

Tissue culture:

The generation of MCF-10Atet cells, a subline of the human mammary epithelial cell line MCF-10A, was described previously³⁸. MCF-10Atet cells were grown at 37 °C in a water vapor saturated 5% CO₂ atmosphere in conventional tissue culture plastic vessels (Sarstedt, Nürnbrecht, Germany) containing DMEM/F12 medium (PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 5 vol% horse serum (PAA, Cölbe, Germany), 1 vol% glutamine (PAN-Biotech GmbH, Aidenbach, Germany), 1 vol% HEPES (PAN-Biotech GmbH, Aidenbach, Germany), 1 vol% penicilline/streptomycine (PAN-Biotech GmbH, Aidenbach, Germany), 250 μ g hydrocortisone (Sigma-Aldrich, Munich, Germany), 50 μ g cholera toxin (Sigma-Aldrich, Munich, Germany), 10 μ g human recombinant epidermal growth factor (R&D Systems, Wiesbaden-Nordenstadt, Germany) and 4.858 mg human recombinant insuline (Actrapid Penfill solution, Novo Nordisk Pharma GmbH, Mainz, Germany). Cells were passaged twice a week or upon reaching confluency and detached by trypsin/EDTA solution. Five hundred cells were plated onto 6 well plates and grown for 24h prior to peptide treatment. For the experiments with Sbc12 cells, we used the stably transfected pool Sbc12ecoR, which expresses the receptor for murine retroviruses. These cells were cultivated as the parental cell line³⁹ and generated using the pQCXIN/ecoR plasmid as described for other cell lines previously¹².

Western blotting

NRAS^{Q61K}-mutant SBC12 melanoma cells were electroporated with the BioRad GenePulser XCell™ in the presence of the indicated concentrations of peptide. Following recovery at 37 °C for 30 min, cells were treated with 1 μ M PLX4032 for 1 h or DMSO as a vehicle control. Subsequently, the cells were harvested, lysed using RIPA buffer and analyzed by Western blotting using the indicated antibodies as described previously¹². Sbc12 cells were lysed in RIPA buffer (50 mM Tris/HCl, pH 7.4; 1% Triton X-100; 137 mM NaCl; 1% glycerin; 1 mM sodium orthovanadate; 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 0.5 mM EDTA; 0.01 μ g/ μ L leupeptin, 0.1 μ g/ μ L aprotinin, 1 mM AEBSF). Lysates were cleared by centrifugation, mixed with sample buffer and analyzed by Western blotting using 10% SDS-PAGE gels as described previously¹² using the following antibodies. Anti-BRAF (F-7) and anti-RAF-1 (C-12) were purchased from Santa Cruz Biotechnology, USA. Anti-phospho-FRA1 (S265; D22B1), anti-FRA1 (D80B4), anti-HSP90 (#4874), anti-phospho-MEK^{1/2} (pS217/221), anti-MEK^{1/2}, anti-p42/p44 MAPK, and anti-phospho-MAPK (pT202/pY204); (ERK^{1/2}) were purchased from Cell Signaling Technologies, USA. Protein concentration determination was performed via BCA assay (Thermo Fisher Scientific, Germany). Equal protein amounts were loaded for PAGE. Blotted

proteins were visualized with a Fusion Solo chemiluminescence reader (Vilber Lourmat, Germany).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

DMF	Dimethylformamide
DCM	Dichloromethane
Fmoc	Fluorenylmethyloxycarbonyl
HATU	O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate
DIPEA	Diisopropylethylamine
Mtt	4-Methyltrityl
2-O-PhiPr	2-Phenylisopropyl
TFA	Trifluoroacetic acid
Mmt	4-Methoxytrityl
NCS	N-chlorosuccinamide
ACN	Acetonitrile
MeOH	Methanol

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Figure 1. Structure of the DIF peptide bound to BRAF (PDB ID 4E26).

The two BRAF monomers are shown as white and yellow surfaces respectively and the DIF sequence is represented by a cyan ribbon and blue carbon atoms. The ATP binding site is filled with a kinase inhibitor in both monomers for perspective.

Figure 2. FAM-TAT-Pep17 impairs the growth of NRAS^{Q61K} driven human Sbc12 melanoma cells.

(A) FAM labeled peptides are taken up by melanoma cells. Sbc12 cells were plated in tissue culture vessels (6-well format) and grown in the presence of the indicated concentrations of FAM labeled TAT peptides. Medium with freshly added peptides were changed every 3 to 4 days. Shown are micrographs taken two weeks after seeding. Cells incubated with the higher concentration accumulate fluorescently labeled peptides, while cells exposed to the lower concentration only display a diffuse autofluorescence. Note that cells treated with the biologically active FAM-TAT-Pep17 not only form smaller colonies, but also tend to display a round morphology, while control peptide exposed cells attached to the substrate are more elongated. (B) Five thousand Sbc12 cells were seeded onto 6-well plates and grown in the presence of the indicated peptide concentrations for two weeks. Medium with freshly added peptides were changed every 3 to 4 days. Cells were stained with Giemsa solution. Shown is a representative result from two independent biological replicates with comparable outcome

Figure 3. Peptide 1 and FAM-TAT-Pep17 impair vemurafenib induced paradoxical ERK pathway signaling

induced by vemurafenib (PLX4032) in NRAS^{Q61K}-mutant SBC12 melanoma cells. **A.** Cells were electroporated with the BioRad GenePulser XCell™ in the presence of the indicated concentrations of peptide 1¹⁶. Following recovery at 37 °C for 30 min, the cells were treated with 1 μM PLX4032 for 1 h or DMSO as a vehicle control. Subsequently, the cells were harvested, lysed using RIPA buffer and analyzed by Western blotting using the indicated antibodies as described previously¹². **B.** NRAS^{Q61K} mutant human Sbc12 melanoma cells were incubated with 3.60 μM of FAM-TAT-Pep6AlaNC3 (control) or FAM-TAT-Pep17 for three days. Four hours prior to harvest, the cells were treated with 1 μM vemurafenib (PLX4032) or the same volume of DMSO as vehicle control. RIPA buffer lysates were subjected to Western blotting using the indicated antibodies. Detection of HSP90 serves as a representative loading control. Note that vemurafenib upregulates the expression and phosphorylation of MEK, ERK and its target FRA1 in FAM-TAT-Pep6AlaNC3 treated control cells, while this response is not observed in the presence of FAM-TAT-Pep17. Shown is one representative experiment out of two independent biological replicates with comparable outcome.

Figure 4. Structure of Peptide 38 bound to BRAF (From PDB ID 4E26).

A. The major binding residue R509 (magenta), smaller (508–513, cyan), and larger macrocyclization sites (505–516, yellow and orange residues replaced with cysteines in peptide **33**) are highlighted. Other important residues for BRAF interaction are labeled including H510, A512 and L515. **B.** Close in view of the arginine handshake motif that provides for a major part of the affinity of the BRAF dimer. R509 (peptide) forms an arginine handshake (anti-parallel binding mode) with Arg509 (BRAF) as observed from the crystal structure. The charge-charge repulsion of the two guanidinium groups is offset by the interaction of the positive charge with the negative charge on the C-terminal end of the α -C helix created by the helix dipole. The ATP competitive kinase inhibitor (from 4E26) is shown as a space filling representation to indicate proximity of the DIF to the catalytic site. **C.** Close in view of the bound conformation of peptide **38** (side chains with green carbons, one letter residue codes) with BRAF (side chains with yellow carbons, three letter residue codes) and illustrating the specific stabilizing non-bonded interactions. Pi-cation, H-bond and salt bridge interactions are shown as light brown, green and dark brown dashed lines respectively.

Table 1.

Structure-activity relationship of BRAF Dimer Interface Peptides

ID	BRAF Residues	Sequence	Kd (μM)
1	503–521	GVLKTRHVNILLFMGYST	3.84 ±0.32
2	503–521 R509H, L515G, M517W	GVLKTHHVNILGFWGYST	NB
3	scrambled	GRINKGRHTFLLVVMYSL	2.96 ± 0.18
4	503–521 L505A	GVARKTRHVNILLFMGYST	3.89 ±0.53
5	503–521 R506E	GVLEKTRHVNILLFMGYST	1.09 ±0.29
6	503–521 R506L	GVLKTRHVNILLFMGYST	0.54 ±0.11
7	503–521 T508D	GVLKDRHVNILLFMGYST	2.2 ±0.83
8	503–521 T508A	GVLKARHVNILLFMGYST	2.8 ±0.29
9	503–521 H510F	GVLKTRFVNILLFMGYST	NB
10	503–521 V511A	GVLKTRHANILLFMGYST	4.75 ±1.7
11	503–521 L514A	GVLKTRHVNIALFMGYST	9.8 ±1.6
12	503–521 L515I	GVLKTRHVNILIFMGYST	4.1 ±1.1
13	503–521 L515homoleucine	GVLKTRHVNIL{HL}FMGYST	1.25 ±0.36
14	503–521 F515D	GVLKTRHVNILLDMGYST	NB
15	503–518	GVLKTRHVNILLFMG	1.88 ±0.36

Table 2.

Ala scan and Truncation studies of BRAF Dimer Interface

ID	BRAF Residues	Sequence	Kd (μ M)
16	504–517	VLKRTRHVNILLFM	5.75 $\pm$ 1.2
17	504–518	VLKRTRHVNILLFMG	0.13 $\pm$ 0.040
18	504–518	VLKRTRHVNILLFMG-NH ₂	0.48 $\pm$ 0.091
19	504–518	Ac-VLRKTRHVNILLFMG	0.8 $\pm$ 0.083
20	504–518 L515homoleucine	VLRKARHVNIL(HL)FMG	0.49 $\pm$ 0.16
21	504–518 L505A	VARKTRHVNILLFMG	0.45 $\pm$ 0.03
22	504–518 R506A	VLAKTRHVNILLFMG	0.36 $\pm$ 0.03
23	504–518 K507A	VLRAHTRHVNILLFMG	ND
24	504–518 R509A	VLKTAHVNILLFMG	2.4 $\pm$ 0.35
25	504–518 H510A	VLKTRAVNILLFMG	2.7 $\pm$ 0.4
26	504–518 N512A	VLKTRHVAILLFMG	NB
27	504–518 I513A	VLKTRHVNALLFMG	2.69 $\pm$ 0.35
28	504–518 L514A	VLKTRHVNIALFMG	1.02 $\pm$ 0.14
29	504–518 F516A	VLKTRHVNILLAMG	0.57 $\pm$ 0.08
30	504–518 M517A	VLKTRHVNILLFAG	0.54 $\pm$ 0.15
31	505–518	LRKTRHVNILLFMG	0.19 $\pm$ 0.13
32	505–518 R506L	LLKTRHVNILLFMG	0.55 $\pm$ 0.105

Table 3.

Structure-activity of cyclic BRAF Dimer Interface Peptides

ID	BRAF Residues	Sequence	Kd (μ M)
33	504–517 cyclo (505, 516) L505C, F516C	VCRKTRHVNILLCM	0.36 $\pm$ 0.32
34	505–518T5080, N512A, I513E	LRKORHVAELLFMG	NB
35	505–518 cyclo(508, 513) T5080, I513E	LRKORHVNELLFMG	0.78 $\pm$ 0.10
36	505–518 cyclo(508, 513) T5080, N512A, I513E	LRKORHVAELLFMG	0.46 $\pm$ 0.04
37	505–518 cyclo(508, 513) I508K, I513E	LRKKRHVNELLFMG	1.89 $\pm$ 0.33
38	505–518 cyclo(508, 513) T508K, N512A, I513E	LRKKRHVAELLFMG	0.061 $\pm$ 0.01