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N-{7-Cyano-6-[4-fluoro-3-({[3-(trifluoromethyl)phenyl]acetyl}amino)phenoxy]-1,3-benzothiazol-2-yl}cyclopropanecarboxamide (TAK632) promotes inhibition of BRAF through the induction of inhibited dimers

Michael Grasso^{1,2,*}, Michelle A. Estrada^{2,*}, Kiara N. Berrios¹, Jeffrey D. Winkler², and Ronen Marmorstein^{1,2}

¹Department of Biochemistry and Biophysics and the Abramson Family Cancer Research Institute, Perelman School of Medicine, University of Pennsylvania, 421 Curie Blvd., Philadelphia, PA 19104, USA

²Department of Chemistry, University of Pennsylvania, 231 S. 34th Street, Philadelphia, PA 19104, USA

Abstract

BRAF^{V600E} is the most common activating mutation in melanoma and patients treated with BRAF^{V600E} inhibitors all develop resistance within 1 year. A significant resistance pathway is paradoxical activation (transactivation) involving BRAF dimers, whereby an inhibitor bound protein subunit allosterically activates the other subunit. We recently reported on bivalent BRAF^{V600E}-selective vemurafenib inhibitors that stabilize an inactive α C-out/ α C-out homodimeric conformation with improved inhibitor potency and selectivity *in vitro*. We set out to extend this strategy to target RAF homo- and hetero-dimers with the pan-RAF inhibitor TAK632 in bivalent configuration. Surprisingly, we find that monovalent TAK632 induces an active α C-in/ α C-in BRAF dimer conformation, while bivalent TAK inhibitors cannot promote BRAF dimers and have significantly compromised potency *in vitro*. These studies uncover the intimate connection between BRAF dimerization and TAK632 mode of inhibition and highlight the importance of understanding the impact of BRAF inhibitors on kinase dimerization.

Graphical abstract

Corresponding Authors Address Correspondence to: Ronen Marmorstein, Ph.D., marmor@upenn.edu, Jeffrey D. Winkler, Ph. D, winkler@sas.upenn.edu.

Author Contributions

M.G. and K.N.B. carried out biochemical and biophysical experiments under the direction of R.M. M.A.E. synthesized compounds under the direction of J.D.W. M.G. prepared figures 1–7. M.G., M.A.E., J.D.W. and R.M. wrote the manuscript. All authors read and approved the manuscript.

*These authors contributed equally to this work.

Ancillary Information

Supporting Information

Information related to the sedimentation equilibrium raw data and residuals, as well as R509A mutation sedimentation velocity experiments and Vem-BisAmide-2 sedimentation velocity experiments. This material is available free of charge via <http://pubs.acs.org>

Introduction

The MAPK pathway is a key regulator of cell proliferation through its control of protein translation and transcription factor regulation via signal transduction. The BRAF kinase within this pathway is a significant oncoprotein, resulting in about 50% of melanomas and a significant number of other cancers including colorectal, ovarian, and lung cancers, among others.^{1–3} A large percentage of BRAF-mutated cancers result from a valine to glutamic acid point mutation at residue 600, stabilizing an active kinase conformation and triggering unregulated progression of the MAPK signaling cascade.³ Due to the prominent driver role of mutant BRAF in melanoma and other cancers, significant effort has been invested towards BRAF inhibitor development, and in particular to inhibiting oncogenic BRAF^{V600E}. While mutant-specific inhibitors such as vemurafenib and dabrafenib have been approved by the FDA to treat patients with metastatic BRAF^{V600E} melanoma,^{4,5} almost all patients develop drug resistance within 6 months of treatment.⁶ These drug resistance pathways usually involve a MAPK pathway reactivation, such as mutation or upregulation of upstream RAS or Receptor Tyrosine Kinases (RTKs) or downstream MEK.⁷ Moreover, while many inhibitors targeting RAF kinases have been developed, most of these compounds have the ability to paradoxically activate (transactivate) BRAF kinases, which typically signal as dimers, at sub-saturating concentrations.^{8–10} Indeed, when activated RAS is present, low concentrations of inhibitor can bind one protomer of a BRAF dimer, allosterically activating the other protomer and hyper-activating the MAPK pathway. This plays a prominent role in inhibitor resistance, as upregulation of upstream kinases such as RAS or RTKs due to resistance pathways can trigger dimerization and, in turn, trigger paradoxical activation.^{11,12} Another form of resistance in BRAF^{V600E} tumors is the expression of a splice variant of BRAF^{V600E} that does not include the RAS binding domain (RBD), leading to enhanced dimerization that is also vulnerable to paradoxical activation.¹³ A recent study reported on the development of inhibitors that can escape paradoxical activation, and these “paradox breaker” inhibitors modify the propyl sulfonamide tail of vemurafenib, which is hypothesized to shift the α C-helix into a dimer destabilizing orientation.^{8,14–16} However, crystal structures of BRAF in complex with paradox breaker inhibitors still form BRAF dimers with α C-helix orientations similar to BRAF kinase dimers with vemurafenib.^{14,18,19} Moreover, these structures also have one BRAF protomer bound to inhibitor while the other is not, and the unbound subunit of the kinase dimer has the α C-helix shifted towards the inward (active) conformation.¹⁴ This raises the possibility of drug resistance developing in patients treated with paradox breaker inhibitors, one of which, PLX8394, is currently in clinical trials (NCT02428712).^{20, 21}

The studies described above highlight the importance of understanding different BRAF inhibitor binding modes and their effects on BRAF dimerization and activation. A prior study characterized several RAF kinase inhibitors that can induce dimerization *in vitro* and in cells, and correlated this to the stabilization of a closed conformation of N and C lobes of the kinase.¹⁷ A more recent study comparing eight diverse RAF inhibitors led to their classification according to their ability to promote an active or inactive α C-helix conformation, α C-in versus α C-out, respectively. The authors determined that the more BRAF mutant specific α C-out inhibitors (such as vemurafenib) are correlated with inhibitor resistance due to negative allostery, in which one subunit of the RAF dimer binds the inhibitor in the α C-out conformation, rendering the other subunit in an inhibitor-free α C-in conformation that promotes paradoxical activation.¹⁸ In contrast, the less BRAF mutant selective pan-RAF α C-in inhibitors can occupy both active sites of a BRAF dimer and are therefore less correlated with drug resistance during treatment.¹⁸

We previously reported on the development of bivalent vemurafenib (Type-I) inhibitors as a novel approach to potently inhibit active BRAF^{V600E} dimers.¹⁹ We found that these inhibitors promote an inactive BRAF^{V600E}/BRAF^{V600E} homodimeric conformation with both protomers in α C-out conformations and with improved vemurafenib potency and selectivity for BRAF^{V600E} *in vitro*.¹⁹ To evaluate the BRAF dimerization and inhibition properties of bivalent inhibitors that contain a monovalent compound that promotes an α C-in conformation, we employed the type-II α C-in inhibitor TAK632.²² We hypothesized that a bivalent TAK inhibitor would further stabilize an inactive dimeric BRAF conformation. Surprisingly, we found that while monovalent TAK632 promotes dimerization and potently inhibits BRAF dimers *in vitro*, bivalent TAK inhibitors cannot induce dimers, and concomitantly reduce inhibitor potency. This study indicates that the promotion of an α C-in/ α C-in BRAF dimer conformation is integral to the ability of TAK632, and likely other Type-II BRAF kinase inhibitors, to inhibit RAF kinases. These studies have implications for the more effective targeting of BRAF dimers with bivalent BRAF inhibitors to address paradoxical activation for more durable treatment of melanoma.

Results

Bivalent TAK inhibitors have reduced potency relative to monovalent inhibitors *in vitro*

The pan-RAF inhibitor TAK632 targets wild-type or mutant BRAF and CRAF with IC₅₀ values in the low nanomolar range.²² We used the crystal structure of BRAF^{WT} complexed with TAK632 (accession code 4KSP) to determine where to link the two TAK632 molecules without compromising TAK632 potency. The TAK632 inhibitor binds both subunits of a BRAF dimer with the α C-helix of both protomers in the α C-in conformation. While the trifluoromethylphenyl moiety of TAK632 is located near the hydrophobic pocket of the binding site, the cyclopropyl amide makes minimal protein contacts, i.e., no interaction between the cyclopropane ring and the protein surface is observed, although there is an interaction between the amide N-H and Cys532 (Figures 1a and 1b). The amide moiety is exposed to solvent, suggesting it would be an appropriate place to link the monomers, with the caveat that the amide N-H bond is retained in the bivalent molecule (Figure 1a). We therefore prepared a series of PEG-linked amide dimers, where n equals the number of PEG

units present in the oxydiacetic acid linker of the dimeric structure (Figure 1c). Superposition of the BRAF^{WT}/TAK632 structure with BRAF^{V600E}/Vem-BisAmide-2, (BRAF^{V600E} bound to a bivalent vemurafenib inhibitor, accession code 5JT2), suggests that the amide-linking site on TAK632 would produce bivalent TAK inhibitors with similar topology as Vem-BisAmide-2 and related compounds (Figure 1d). The distance between the two TAK632 molecules within a BRAF dimer of the BRAF^{V600E}/Vem-BisAmide-2 structure would be predicted to be about 10 Å. Based on this docking exercise, we synthesized a series of bivalent TAK-n-TAK molecules where n=0, 2, 3, 4, and 6 to account for various possible protein dimer orientations (Figure 1e). As a control, we also made two compounds that contain one TAK molecule and parts of the linker to evaluate any adverse effects made by removing the cyclopropane group (Figure 5).

The requisite dimers were prepared by reaction of the known TAK aminobenzothiazole **1** with the requisite oxydiacetic acids **2–6** in the presence of BEP and DIPEA in DMF, in which the tether length was increased by incremental addition of ethylene glycol moieties to generate the TAK-n-TAK series, in which n represents the number of ethylene glycol moieties in the linker between the two TAK ligands. The inhibitor potency of each molecule was then evaluated *in vitro* against both BRAF^{WT} and BRAF^{V600E} using an ELISA assay that measures the level of phosphorylation of GST-tagged MEK by purified BRAF kinase domain. While all inhibitors showed comparable potency against BRAF^{WT} and BRAF^{V600E}, their inhibitory potencies were 15- to 400-fold reduced relative to monovalent TAK632 (Figure 2a and 2b). Compounds TAK-2-TAK and TAK-4-TAK showed the greatest potencies of the bivalent inhibitors, with IC₅₀ values of 132 nM and 90.2 nM, respectively, against BRAF^{WT} and 73.9 nM and 73.8 nM, respectively, against BRAF^{V600E}. The other bivalent TAK inhibitors (TAK-0-TAK, TAK-3-TAK and TAK-6-TAK) had IC₅₀ values ranging from 168 nM to 732 nM. In comparison, monovalent TAK632 had IC₅₀ values of 3.23 nM and 4.46 nM against BRAF^{WT} and BRAF^{V600E}, respectively. These experiments reveal that although the bivalent TAK inhibitors show some dependency on linker length, they are considerably less potent than monovalent TAK632 and therefore likely binding BRAF in a different mode than bivalent Vem-BisAmide-2 and related compounds.

Monovalent TAK inhibitors promote BRAF dimers while bivalent TAK inhibitors do not

Our previous studies revealed that bivalent vemurafenib inhibitors promoted an inactive “face-to-face” αC-out/αC-out BRAF dimer configuration that differed significantly from the “side-to-side” active αC-in/αC-out BRAF dimer configuration as bound to monovalent vemurafenib or αC-in/αC-in BRAF dimer configuration not bound to inhibitor.¹⁹ To determine if bivalent TAK inhibitors also promoted BRAF dimers, we performed analytical ultracentrifugation (AUC) sedimentation velocity experiments to compare the oligomeric state of BRAF as a function of added TAK inhibitors. We first utilized an R509H BRAF mutant protein that disrupts the side-to-side active dimer interface to promote the formation of BRAF monomers.¹¹ As expected, unliganded BRAF^{R509H} migrated with a sedimentation coefficient of ~ 3 corresponding to an apparent protein monomer (Figure 3a). Surprisingly, however, the addition of a molar excess of bivalent TAK inhibitors did not alter the apparent monomer migration position of BRAF^{R509H}, irrespective of linker length (Figures 3a – 3c). The bivalent TAK inhibitors were therefore unable to shift BRAF into an inactive dimeric

configuration as anticipated. This differed from chemically linked Vemurafenib inhibitors such as Vem-BisAmide-2, which were able to shift BRAF^{V600E/R509H} into a dimeric conformation in solution (Figure S1).

In contrast to the effect of adding bivalent TAK inhibitors to BRAF^{R509H}, the addition of a molar excess of monovalent TAK632 to BRAF^{R509H} led to the formation of an apparent BRAF^{R509H} dimeric species (sedimentation coefficient of ~ 4, Figure 3a – 3c). While the literature demonstrates that TAK632 can induce dimers at lower concentrations than vemurafenib²³, the fact that TAK632 can induce dimers *in vitro* despite a R509H point mutation that is known to disrupt dimers was unexpected. These studies demonstrate that monovalent TAK632 actively promotes the formation of BRAF dimers.

To determine whether higher concentrations of the bivalent TAK inhibitors are able to shift BRAF^{R509H} into a dimeric configuration, we titrated 20 μ M protein with 25 – 200 μ M TAK-4-TAK and found that even the highest concentration of bivalent inhibitor was unable to fully shift the protein into a dimeric configuration, although at the highest concentration of TAK-4-TAK (200 μ M), BRAF^{R509H} gives a more broad sedimentation curve, indicating that at these high concentrations the protein/inhibitor complex begins to shift towards a dimeric species (Figure 4a). Consistent with the results above, we also demonstrated that BRAF^{WT} and BRAF^{V600E} form dimeric species in the presence of monovalent TAK632 and form either monomers or mixed monomer/dimer populations in the presence of bivalent TAK inhibitors (Figures 4b and 4c). To confirm these findings, we ran sedimentation equilibrium experiments of BRAF^{R509H} in the absence and presence of monovalent TAK632 and bivalent TAK inhibitors. Log plots of the data are shown in Figure 4d, where the slope of the line is proportional to the estimated molecular weight of the species in solution. Sedimentation equilibrium curves and residuals are shown in Figure S2. BRAF^{R509H}/TAK632 gave an ideal molecular weight fit of ~69 kDa, aligning with the simulated dimer of ~70 kDa. In contrast, BRAF^{R509H}/TAK-4-TAK gave an ideal molecular weight fit of ~40kDa, aligning with the simulated monomer of ~35 kDa. BRAF^{R509H} without inhibitor also aligns with the simulated monomer, giving an ideal molecular weight fit of ~36 kDa. Taken together, the observation that monovalent TAK632 inhibitors promote the formation of BRAF dimers and that the bivalent TAK inhibitors cannot, coupled with our earlier findings that monovalent TAK632 is much more potent than bivalent TAK for BRAF inhibition (Figure 2), leads to the conclusion that TAK632 promotes inhibition of BRAF through the induction of inhibited dimers.

The bivalent nature of the linked TAK inhibitors is required to reduce inhibitor potency and to promote BRAF monomers

To determine whether the two TAK632 ligands or the glycol linker was responsible for promoting the formation of the BRAF monomers, we prepared two compounds with the linker intact and only one TAK632 molecule (TAK-L and TAK-L-C, Figure 5a). TAK-L, included the PEG portion of the linker, while TAK-L-C included the PEG and the 1,3-thiazole-2-amide moiety of the second TAK632 molecule as a cap. Coupling of the TAK molecule **1** (Figure 1e) with commercially available 2-(2-methoxyethoxy)-acetic acid **12** led to the formation of **13 TAK-L** (see Supplementary Information for Details). The TAK-L-C

14 was prepared from 3,6,9-trioxaundecandioic acid **3**, first by anhydride formation with DCC, followed by ring opening with 2-aminothiazole, and coupling of the TAK molecule **1** with the resulting monoacid intermediate to give **14 (TAK-L-C)**. Both molecules were evaluated in ELISA kinase activity assays and sedimentation velocity experiments to assess the effect of these two linker regions on TAK potency and the ability to promote BRAF dimers, respectively. Dose response kinase inhibition experiments demonstrated that TAK-L had similar inhibitor potency to TAK632, with IC₅₀ value of 5.75 nM and 7.11 nM, respectively, and TAK-L-C showed only about a 4-fold reduced potency (20.3 nM) relative to TAK632 (Figure 5b). In contrast, the bivalent TAK inhibitors, TAK-2-TAK and TAK-4-TAK, showed about a 12-fold reduction in potency (> 85 nM) relative to TAK632 (Figure 5b). These data demonstrate that the second TAK632 ligand in the bivalent TAK inhibitor plays a significant role in the reduced potency of the bivalent TAK inhibitors relative to monovalent TAK632. These results also indicate that linker placement and removal of the cyclopropane group does not significantly hinder the ability of the bivalent inhibitors to inhibit BRAF relative to monovalent TAK632.

Sedimentation velocity experiments with BRAF^{R509H} in the presence of monovalent TAK-L and TAK-L-C or the bivalent TAK-2-TAK, reveal that the TAK inhibitors containing linkers and a single TAK632 ligand promoted the formation of protein dimers, while TAK-2-TAK could not alter the oligomerization state upon binding (Figure 5c), as previously shown (Figure 3a). These data demonstrate that the second TAK632 ligand in the bivalent TAK inhibitor plays a driving role in preventing the formation of inhibited BRAF dimers, which appears to be correlated with the reduced potency of the bivalent TAK inhibitors relative to monovalent TAK632.

Bivalent TAK inhibitors display distinct BRAF properties

To further explore the mechanism by which bivalent TAK inhibitors bind BRAF, we performed a limited proteolysis experiment in which trypsin was added to BRAF^{WT} in the presence and absence of TAK632, TAK-2-TAK, TAK-L-C, and vemurafenib (Figure 6A). When no ligand is present (lane 1), the major digested band (species A), is very close in size to undigested BRAF, with the appearance of two smaller minor bands (species B and C). In the presence of TAK632 and TAK-L-C (lanes 2 and 3 respectively), BRAF^{WT} species A becomes a minor band, while species B and C become major bands. In contrast, in the presence of TAK-2-TAK (lane 4), BRAF^{WT} had a digestion pattern resembling that of unliganded BRAF^{WT}, producing BRAF^{WT} species A as a major band but with slightly larger amounts of species B and C. In the presence of vemurafenib (lane 5), BRAF^{WT} produces species A as the major band, with negligible amounts of species B and C. These results demonstrate that in the presence of the bivalent TAK-2-TAK, BRAF has a digestion pattern that is somewhere in between BRAF^{WT} alone and monomeric TAK632/TAK-L-C. These observations indicate that bivalent TAK inhibitors cannot fully promote a conformation allowing for degradation to species B and C, whereas monovalent TAK molecules can.

To further dissect the mode of bivalent TAK inhibitor binding to BRAF, we performed Differential Scanning Calorimetry (DSC) experiments in which BRAF^{WT} protein (37 μM)

was heated in the presence and absence of ligands to determine melting temperatures (Figure 6B). When no ligand is present (black), we observe a single melting temperature of 37.84 °C, which we interpret as melting of a BRAF^{WT} monomer. In the presence of TAK632 or TAK-L-C (125 μM) (violet and cyan, respectively), we observe single melting temperatures of 68.74 °C and 63.29 °C, respectively, which we interpret as melting of BRAF^{WT} dimers bound to monovalent TAK inhibitors. In contrast, in the presence of TAK-2-TAK (125 μM) (pink), two broad melting temperatures are observed of 38.72 °C and 59.6 °C, and titration of TAK-2-TAK between 75–250 μM produces more of the higher melting temperature species at the expense of the lower melting temperature species. We interpret this observation to indicate that while bivalent TAK inhibitors do have a capacity to promote BRAF dimers at high concentration of bivalent inhibitor, they do so significantly more poorly than monovalent TAK632. Taken together, both the limited proteolysis (Figure 6A) and DSC (Figure 6B) studies reveal that bivalent TAK inhibitors promote BRAF species in solution that act as a combination of both unbound BRAF and BRAF bound to monovalent TAK, indicating that the bivalent TAK inhibitors cannot fully stabilize the dimeric TAK632-mediated BRAF configuration.

Trp450, Arg506 and the αC-helix play a significant role in dimer formation via TAK binding

Given our surprising finding that the monovalent TAK632 inhibitor promotes formation of αC-in/αC-in BRAF dimers, even in the presence of the R509H dimerization defective mutant, we set out to better understand the nature of the BRAF dimer that is stabilized by monovalent TAK632. R509 is able to stabilize the active “side-to-side” αC-in/αC-in configuration by making hydrogen bond interactions with backbone carbonyls of T508 and R506.^{11, 19} To assess what additional changes are necessary to facilitate TAK632-induced dimerization, we superimposed different subunits of the BRAF^{WT}/TAK632 (accession code 4KSP)²², BRAF^{V600E}/AZ628 (accession code 4G9R)²⁴ and BRAF^{R509H}/AZ628 (accession code 4RZW)¹⁸ crystal structures. AZ628 is another Type-II, αC-in inhibitor, and was also shown to be able to induce dimerization in the presence of an R509H mutation, however at a higher concentration.²⁴ This superposition revealed that the inhibitor complexes with BRAF^{WT} and BRAF^{V600E} contain highly super imposable αC-in/αC-in configurations (RMSD of 0.596 Å² for BRAF^{WT}/TAK αC segments and 0.463 Å² for BRAF^{V600E}/AZ628 αC segments), while the BRAF^{R509H}/AZ628 structure contains more variable αC-in configurations (RMSD of 1.713 Å² for corresponding αC segments) where one of the αC segments orients further away from the αC-out configuration while the other moves closer towards the αC-out configuration (as seen in the BRAF^{V600E}/Vem complex, Figure 7a). This observation suggests that while the R509H mutation destabilizes the active αC-in/αC-in dimer and favors an inactive, αC-out configuration, Type-II inhibitor binding is able to compensate for the destabilizing R509H mutation to further strengthen the dimer by biasing the kinase towards an αC-in/αC-in dimer configuration.

In comparing the three crystal structures described above, we noted that W450 participates in van der Waals interactions with the aliphatic region of R509 but that W450 adopts a distinct conformation in one of the protomers of the BRAF^{R509H}/AZ628 structure, which appears to be facilitated by a pi-stacking interaction with the R509H mutation of the other protomer (Figures 7b and 7c), and accompanied by a movement of D448 to hydrogen bond

to W450 of the opposing subunit (Figure 7d). Reinforcing the importance of W450 for active BRAF dimer formation, a W450A mutation was previously demonstrated to impair transactivation in cells.^{10, 25} These results indicate that W450 plays a critical role in the ability of TAK632 (and possibly other Type-II α C-in inhibitors such as AZ628) to induce dimerization.

R506 is another residue previously noted to play a significant role in inhibitor-induced dimerization based on the unique conformations that it adopts in co-crystal structures of BRAF bound to α C-in (ie. AZ628) and α C-out (ie. vemurafenib) inhibitors.¹⁸ Specifically, R506 adopts an “in” position in the BRAF^{R509H}/AZ628 structure, but adopts an “out” position in BRAF^{V600E}/vemurafenib (Vem) (3OG7, yellow) structures (Figure 7e). R506 lies close to W450, and the distinct W450 shift present in the one BRAF^{R509H}/AZ628 protomer that shifts closer to the R506 “out” conformation, further supports the integral role of R506 in dimerization (Figure 7f).

To evaluate the effect of W450 and R506 on TAK-induced dimerization, we performed sedimentation velocity experiments with BRAF^{W450A}, BRAF^{R506A}, BRAF^{W450A/R509H}, BRAF^{R506A/R509H}, and BRAF^{R506A/W450A/R509H} mutants alone and in the presence of TAK632 (Figure 8a–8b). We found that TAK632 promotes the complete dimerization of BRAF^{R506A} and BRAF^{W450A}, but TAK632 with the double mutants of BRAF^{R509H/W450A} and BRAF^{R506A/R509H} showed a peak that is in between that of a monomer and a dimer. The peaks also appear to be slightly broader than those of species that sediment as complete monomers or dimers. We hypothesize that these mutants are able to disrupt TAK632-induced dimerization, however not completely, thus giving a mixed monomer/dimer peak. The triple mutant BRAF^{R509H/R506A/W450A} in the presence of TAK632 sediments less broadly and more closely to that of a monomer peak, indicating that the combination of all three mutations disrupts TAK632 induced dimerization more than any of the individual mutations. We confirmed these results with sedimentation equilibrium measurements (Figure 8c), where BRAF^{R509H/R506A} gives an estimated ideal molecular weight of 58,000 kDa while BRAF^{R509H/W450A} gives an estimated molecular weight of 57,000 kDa (Raw curves and residuals are shown in Figures S2 and S3.) BRAF^{R509H/R506A/W450A} leads to the smallest molecular weight of the mutants, 47,000 kDa, indicating that while TAK632 binding does still induce dimerization slightly, the ability to do so is dramatically decreased due to mutation of the residues that mediate dimerization. While each of these mutants alone is not enough to prevent TAK632-induced dimerization, combining them causes conformational changes in the active dimer and combining all three prevents the majority of molecules in solution from forming dimers. Taken together, these studies indicate that R509, W450 and R506 play important roles in facilitating the active α C-in/ α C-in BRAF dimer as well as TAK632-induced dimerization. The observation that TAK632 still efficiently promotes dimers of the single BRAF^{W450A}, BRAF^{R506A}, and BRAF^{R509H} mutants but not the BRAF^{R509H/R506A/W450A} mutant suggests that all three residues play a coordinated role in “side-to-side” α C-in/ α C-in configuration dimerization, which is further reinforced by TAK632 binding.

Type II α C-in and Type I α C-out inhibitors promote BRAF dimers and monomers, respectively

To assess whether other Type II α C-in inhibitors are able to induce BRAF dimerization in solution, we performed sedimentation velocity experiments with sorafenib, another well-known Type II, α C-in inhibitor.^{18,26} As shown in Figure 8d, sorafenib behaves similarly to TAK632, promoting dimerization despite the R509H mutation, further reinforcing the finding that Type II α C-in inhibitors function by stabilizing the active “side-to-side” α C-in/ α C-in BRAF dimer configuration. To explore the effects of a Type I α C-out inhibitor, we used vemurafenib as a model α C-out inhibitor. We found that BRAF^{WT} monomers were stabilized and BRAF^{V600E} dimers were disrupted upon addition of vemurafenib (Figure 8e). Taken together, Type II α C-in inhibitors appear to promote BRAF dimerization, while Type I α C-out inhibitors such as vemurafenib promote a disruption of the BRAF dimer. TAK632 therefore relies on its ability to induce dimerization for effective inhibition, unlike α C-out inhibitors such as vemurafenib.

Discussion and Conclusions

In this study, we demonstrate that the induction of an α C-in/ α C-in configuration is integral for potent TAK632 inhibition. The mutational and analytical ultracentrifugation analyses further highlight the importance of R509, W450, R506, and likely also D448 in mediating this dimeric BRAF conformation. The analysis that we present here with the other Type II α C-in inhibitors AZ628 and sorafenib suggests that these findings extend to the broader family of α C-in inhibitors. Coupled with the potent BRAF inhibitory activity and absence of paradoxical activation activity of such inhibitors, these findings support the conclusion that TAK632 and related inhibitors promote inhibition of BRAF through the induction of inhibited dimers.

While previous studies have highlighted the importance of R509 in stabilizing α C-in/ α C-in BRAF dimers, we have further analyzed the molecular basis of stabilization of this dimeric BRAF configuration, concomitant with positioning the C-helix in the α C-in configuration. We demonstrate that W450, R506 and likely also D448 play important roles in this activity. Other studies have also proposed the importance of R506 in mediating BRAF dimer formation, and we have demonstrated that it plays a similar role in inhibitor-induced dimerization as W450.¹⁸

In contrast to monovalent TAK632, the less potent bivalent TAK inhibitors appear unable to promote the α C-in/ α C-in dimeric configuration. This leads to BRAF monomers being the predominant species, while monovalent TAK632 is able to induce dimerization upon binding. Comparison of the BRAF activities and multimerization states of BRAF complexes with bivalent TAK inhibitors and monovalent TAK632 inhibitors with attached linkers reveals that the second TAK632 molecule of the bivalent TAK inhibitors plays a particularly important role in the reduced BRAF kinase activity and promotion of the monomeric BRAF state. The control inhibitors TAK-L and TAK-L-C also demonstrate that linker placement and removal of the cyclopropane group does not affect active site binding, indicating a more complex mechanism. Although our data implies that the reduced inhibitor activity of the bivalent TAK inhibitors is correlated with their inability to promote BRAF dimers, the

molecular basis for how the second TAK632 ligand of the bivalent TAK inhibitor destabilizes the BRAF dimer configuration that is promoted by TAK632 is unclear. Limited proteolysis and DSC experiments suggest that while bivalent TAK inhibitors can mediate a BRAF configuration that is similar to that of TAK632-bound BRAF, their affinities for these sites are notably decreased. We propose that bivalent TAK inhibitors have significantly reduced affinities for BRAF because they are unable to assume stable dimeric BRAF configurations. We have extensively attempted crystallization of these molecules with BRAF^{WT} and were unsuccessful, and we hypothesize this is due to the second molecule of TAK binding loosely, making the species in solution heterogeneous and dynamic, hindering any ability to crystallize.

In previous studies, we demonstrated that bivalent vemurafenib inhibitors promote inactive BRAF^{V600E}/BRAF^{V600E} homodimeric conformations with both protomers containing α C-out conformations. These bivalent inhibitors also displayed improved potency and selectivity for BRAF^{V600E} *in vitro*.¹⁹ Interestingly, we found that bivalent vemurafenib inhibitors were able to induce the same, face-to-face α C-out/ α C-out BRAF dimeric configurations, independent of linker length. This was not the case with bivalent TAK inhibitors in this study, in which we found that the bivalent TAK inhibitors cannot promote BRAF dimers, resulting in reduced potency relative to monovalent TAK632. To understand the molecular basis for why bivalent TAK inhibitors are unable to induce the same dimer face-to-face α C-out/ α C-out BRAF dimeric configuration promoted by bivalent vemurafenib inhibitors, we overlaid individual BRAF molecules bound to TAK632 with the “face-to-face” conformation of BRAF bound to the bivalent Vem-BisAmide-2 inhibitor (Figure 9A). Although this modeling exercise does not reveal any steric clashes between the BRAF molecules in the modeled TAK632-bound inactive dimeric configuration, we observe different configurations of the Vem-BisAmide-2 and TAK632-bound BRAF proteins that could destabilize TAK632-bound BRAF dimers in this BRAF dimer configuration (Figure 9B). Specifically, we note that while Vem-BisAmide-2 binding favors the activation segment to flip outwards into an active conformation (cyan), the activation segment of BRAF bound to TAK632 molecules favors an inactive, inward activation segment conformation (magenta). While the activation segment is mostly unresolved in the BRAF/TAK632 structure, this dynamic region could form steric clashes with the activation segment of another BRAF molecule, making the “face-to-face” dimeric conformation energetically unstable. We therefore propose that it is not possible for bivalent TAK inhibitors to induce an inactive, “off-state” BRAF dimer configuration, and this is likely true with other α C-in inhibitors such as sorafenib and AZ628. Instead, we hypothesize that pan-RAF inhibitors that favor the α C-out conformation will be more amenable to the preparation of bivalent inhibitors with improved BRAF potency and with the ability to counteract transactivation of RAF^{WT}/RAF^{WT} homodimers and RAF^{WT}/BRAF^{V600E} heterodimers in melanoma and other BRAF-associated cancers. Interestingly, the pan-RAF inhibitors reported to date all appear to stabilize the active α C-in conformation. We propose that a solution to this is to prepare bivalent BRAF inhibitors with highly potent BRAF^{V600E}-specific α C-out promoting inhibitors that still retain appreciable affinity for BRAF^{WT}. Such inhibitors could be molecules such as Dabrafenib and BI 882370, which inhibit BRAF^{WT} and CRAF^{WT} with potencies similar to pan RAF inhibitors such as TAK632.²⁷ Another possibility is a

purinylpyridinylamino-based BRAF inhibitor that is BRAF^{V600E}-specific, but binds two molecules of a BRAF^{WT} side-to-side dimer with two α C-out configurations.²⁸ Taken together, these studies highlight the importance of understanding the impact of BRAF inhibitors on kinase dimerization to effectively target RAF^{WT}/RAF^{WT} homodimers and RAF^{WT}/BRAF^{V600E} heterodimers with bivalent pan-RAF inhibitors to target paradoxical activation for more durable treatment of melanoma.

Experimental Section

1. Plasmids.

Proteins used for analytical ultracentrifugation sedimentation velocity

experiments.—DNA encoding the BRAF kinase domain residues 448–723 containing 16 solubilizing mutations (I543A, I544S, I551K, Q562R, L588N, K630S, F667E, Y673S, A688R, L706S, Q709R, S713E, L716E, S720E, P722S, and K723G) was ordered from Epoch Biolabs and cloned into a Pet28a(+) vector encoding an N-terminal 6XHis Tag and a thrombin cleavage site between the protein and the tag. This construct was used as a template to create His-tagged BRAF^{V600E}, BRAF^{R509H}, BRAF^{R506A}, BRAF^{W450A}, BRAF^{W450A/R509H}, BRAF^{R506A,R509H}, and BRAF^{R506A,R509H,W450A} mutants (each harboring the 16 stabilizing mutations noted above). These proteins were used in analytical ultracentrifugation sedimentation velocity experiments.

Proteins used for kinase inhibition assays.—DNA encoding the BRAF kinase domain residues 442–724 was used as a template and cloned into a Pfastbac dual vector with mouse p50^{cdc37} full length as an expression chaperone for protein expression in baculovirus infected Sf9 insect cells. An N-terminal 6X-His tag was inserted into the plasmid, and this plasmid was used as a template to create mutant BRAF^{V600E}. Full length human MEK1 with an N-terminal GST fusion tag and a C-terminal His tag in a pGex-3t vector was provided by Dr. Michael Olson (Beatson Institute for Cancer Research, Glasgow, UK) and was used as the substrate for the *in vitro* kinase assays.

2. Protein Purification.

Proteins used for analytical ultracentrifugation sedimentation velocity, limited proteolysis, and differential scanning calorimetry experiments.—His-tagged BRAF proteins were produced as previously described.¹⁹ In brief, proteins were expressed in (DE3)RIL bacterial expression cells at 37 °C and induced with 1mM IPTG overnight at 18 °C, spun down the next day, and lysed in lysis buffer (50mM potassium phosphate pH 7.0, 250mM NaCl) with 1mM PMSF and DNaseI. The lysate was spun down at 19000 rpm for 20 minutes, and the supernatant was added to 7mL of TALON metal affinity resin (Takara) and left to incubate at 4 °C for 1 hr. The supernatant was eluted via gravity column, and the resin was washed with 1 L of lysis buffer with 10 mM imidazole. The BRAF proteins were eluted with lysis buffer supplemented with 250 mM Imidazole. Protein was dialyzed into dialysis buffer (50 mM potassium phosphate pH 7.0, 5 mM EDTA pH 7.5, 1mM DTT (Dithiothreitol)) overnight and then applied to a 5 mL SP Sepharose cation exchange column followed by washing in dialysis buffer and elution in 50 mM potassium phosphate pH 7.0, 1 M NaCl, and 1 mM DTT. Peak fractions were run on an SDS-PAGE

gel, pooled, concentrated, and applied to a Superdex S200 gel filtration column in a final buffer of 20 mM HEPES pH 7.0, 150 mM NaCl, 5% Glycerol and 10 mM DTT. Protein was concentrated to 5–10 mg/mL, flash frozen in liquid nitrogen, and stored in –80 °C freezer for future use.

Proteins used for kinase inhibition assays.—BRAF^{WT} and BRAF^{V600E} were overproduced as N-terminally His-tagged proteins in insect cells essentially as previously described.¹⁹ Briefly, protein constructs were coexpressed with p50^{cdc37}, pelleted, suspended in lysis buffer 2 (25 mM Tris pH 8.0, 250 mM NaCl, 5 mM Imidazole and 10% glycerol) treated with Complete EDTA-free protease inhibitor cocktail tablets (Roche) and DNaseI, lysed, centrifuged at 19,000 rpm for 30 minutes, and added to TALON metal affinity resin and incubated for 1 hour at 4 °C. The protein on the resin was washed extensively with 1 L of lysis buffer 2 and eluted with 25 mM Tris pH 7.5, 250 mM NaCl, 250 mM imidazole, and 10% Glycerol. The protein was diluted into a low salt buffer containing 25mM Tris pH 8.0, 1 mM EDTA and 1 mM DTT and run on an SP Sepharose cation exchange column, and eluted with a salt gradient from 50 mM NaCl to 1 M NaCl. Peak fractions were run on an SDS-PAGE gel and fractions containing protein were pooled, concentrated, and applied to a Superdex S200 gel filtration column and stored in a final buffer of 25 mM Tris pH 8.0, 300 mM NaCl, 1 mM DTT, and 10% glycerol. Protein was concentrated to ~0.5 mg/mL and flash frozen in liquid nitrogen and stored for later use in a –80 °C freezer.

GST-MEK1 fusion protein used as a substrate in ELISA assays was prepared essentially as described previously.¹⁹ Briefly, the protein was expressed in (DE3) RIL cells at 37 °C and induced with 0.5 mM IPTG at 15 °C overnight. The cells were harvested and resuspended in lysis buffer 3 (20 mM HEPES at pH 7.0, 500 mM NaCl, 10 mM BME, 10 mM imidazole and 5% glycerol) supplemented with 1mM PMSF and DNaseI. The lysate was sonicated and spun down at 19,000 rpm for 30 minutes and the supernatant was added to Ni-NTA resin and incubated for 1 hr at 4 °C. The resin was washed extensively with lysis buffer 3 with 20 mM Imidazole instead of 10 mM and eluted with lysis buffer 3 supplemented with 250 mM imidazole. Eluted protein was concentrated and loaded onto a Superdex S200 16/60 gel filtration column into a final buffer of 20 mM HEPES pH 7.0, 150 mM NaCl, 10 mM BME and 5% glycerol. The protein eluted off the sizing column in two separate populations, and the second peak was collected, concentrated to ~20 mg/mL, and flash frozen in liquid nitrogen and stored in –80 °C freezer for future use.

***In Vitro* Kinase Assay.**

Compound inhibition of BRAF^{WT} and BRAF^{V600E} were performed using an ELISA assay described previously.¹⁹ Briefly, GST-MEK fusion protein was diluted 3:1000 in Tris-buffered saline (25 mM Tris pH 7.5, 140 mM NaCl) treated with 0.05% Tween-20 (TBST), and diluted MEK was added to each well of a glutathione coated 96-well plate (Pierce #15240) and incubated at room temp for 1 hr with shaking. BRAF was diluted from frozen stocks (1:500 dilution for BRAF^{WT} and 1:1000 dilution for BRAF^{V600E}) in 50 mM HEPES pH 7.0 and 50 mM NaCl. 2 µL of desired concentration of inhibitor was added to 100 µL of diluted BRAF in a 96 well “V” bottom plate (Corning #2897) and the inhibitor/protein mixture was incubated for 1 hr at room temp. Glutathione plates were washed extensively

with TBST and the protein-inhibitor mixture was added to the plate with a 100 μ M final concentration of ATP in a buffer containing 50 mM HEPES pH 7.0, 200 mM NaCl, and 20 mM $MgCl_2$. The plate was incubated at 37 °C for 30 minutes. The reaction was washed from the plate and the plate was again washed with TBST. A 1:8000 dilution of primary antibody (p-MEK $\frac{1}{2}$ (S217/S221) rabbit antibody (cell signaling)) in TBST treated with 0.5% BSA was added to the plate and incubated for 1 hr with shaking. The plate was then treated with multiple TBST washes and then incubated with a 1:10,000 dilution of secondary antibody (goat anti-rabbit IgG (H+L)-HRP (BioRad)) in TBST treated with 0.5% BSA for 1 hr with shaking. The plate was washed extensively with TBST and Supersignal ELISA Pico Chemiluminescent Substrate (Pierce #37069) was added. The plate was read on a Promega GloMAX 96 Microplate Luminometer. Each curve was repeated in duplicate or triplicate, normalized using GraphPad Prism by selecting the largest value as the maximum and the lowest value as the minimum, and used to calculate IC₅₀ values by using a log (inhibitor) vs response fit on Prism 5.0 (GraphPad). Error bars are indicative of the SEM of each point, and 95% confidence intervals are listed in the figure legends as calculated by GraphPad Prism.

3. Analytical Ultracentrifugation (AUC).

Sedimentation velocity AUC was performed with a Beckman Optima XL-I at 42,000 rpm. Data were obtained over a period of ~15 hours of centrifugation at 20 °C by monitoring absorbance. Previously frozen stocks of BRAF and all corresponding mutations (R509H, V600E, R506A, W450A, R506A/R509H, R509H/W450A, and R506A/R509H/W450A) were thawed and diluted to ~10–20 μ M depending on the experiment in AUC buffer (25 mM Tris pH 7.5, 150 mM NaCl) and inhibitor was added to the desired final concentration by adding 20 μ L of stock concentration of inhibitor in 100% DMSO to 430 μ L of protein to give a final DMSO concentration of 4.44%. Samples run without inhibitor had 20 μ L of DMSO added to give the same 4.44% DMSO concentration as a control. Data were analyzed using SEDFIT to calculate a continuous c(s) distribution with a frictional coefficient set to 1.20, and data were graphed using GraphPad Prism.

Sedimentation Equilibrium AUC was performed with the same Beckman Optima XL-I at three speeds (9,000 rpm, 12,000 rpm, and 18,000 rpm) at three different concentrations (20 μ M, 10 μ M, and 5 μ M) of BRAF^{R509H} supplemented with a 2:1 molar ratio of inhibitor to protein at each concentration. AUC buffer from sedimentation velocity experiments were used in sedimentation equilibrium experiments. Data were analyzed using heteroanalysis to calculate an ideal fit molecular weight of the species, and log plots of the data were subsequently graphed using GraphPad Prism. Log plots were calculated using the 12,000 rpm data of each set. Ideal monomer and dimer fits were calculated using heteroanalysis. Raw data curves and residuals are shown in Figures S2 and S3.

4. Limited Proteolysis.

98 μ L of 29 μ M BRAF^{WT} in LP Buffer (20 mM HEPES pH 7.0, 150 mM NaCl) were added to eppendorf tubes with 1 μ L of 0.5 mg/mL trypsin (Sigma-Aldrich, T1426–50MG) and 2 μ L of either DMSO or inhibitor dissolved in 100% DMSO to give a final inhibitor concentration of 200 μ M. Inhibitors TAK632, TAK-4-TAK, TAK-L-C, and vemurafenib

were tested, as well as a control in which no trypsin was added. After 30 min. of protease treatment, 20 μ L of the reaction mixture was removed and added to 5 μ L of 5X SDS loading buffer, boiled and run on a 13.5% Acrylimide gel using SDS-PAGE, followed by staining using Coomassie blue.

5. Differential Scanning Calorimetry (DSC).

BRAF^{WT} was diluted to a final concentration of 37 μ M in DSC Buffer (20 mM HEPES pH 7.0, 150 mM NaCl). 50 μ L of either DMSO or inhibitor dissolved in 100% DMSO was added to 450 μ L of BRAF^{WT} and degassed for 3 minutes. The protein/inhibitor or protein/DMSO mixture was then added to a MicroCal VP-Capillary DSC (Malvern) and blanked with 450 μ L of DSC Buffer and 50 μ L of DMSO. The protein (sample) and buffer (blank) were both heated from 10 $^{\circ}$ C to 90 $^{\circ}$ C with a scan rate of 60 $^{\circ}$ C/hour and a filtering period of 10 seconds. The difference in heat required to raise the temperature of the sample as compared to the blank is measured as a function of temperature. The data was plotted using Origin 7.

6. Small Molecule Inhibitors.

PLX4032 (vemurafenib) was purchased from Santa Cruz Biotechnology (cat# sc-364643). Sorafenib was purchased from Santa Cruz Biotechnology (cat# sc-220125). TAK632 was purchased from BioVision Inc. (cat# 2473–5).

7. General Chemistry Information.

Solvents used for extraction and purification were HPLC grade from Fisher. Unless otherwise indicated, all reactions were run under an inert atmosphere of argon. Anhydrous tetrahydrofuran, diethyl ether, and toluene were obtained via passage through an activated alumina column. Merck pre-coated silica gel plates (250 mm, 60 F254) were used for analytical TLC. Spots were visualized using 254 nm ultraviolet light, with either anisaldehyde or potassium permanganate stains as visualizing agents. Chromatographic purifications were performed on Sorbent Technologies silica gel (particle size 32–63 microns). ^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, or 360 MHz and 90 MHz, respectively, in CDCl_3 , $\text{DMSO}-d_6$ or CD_3OD on a Bruker AM-500, a DRX-500, or a DMX-360 spectrometer. Chemical shifts are reported relative to internal chloroform (δ 7.26 for ^1H , δ 77.0 for ^{13}C), $\text{DMSO}-d_6$ (δ 2.50 for ^1H , δ 39.5 for ^{13}C), or CD_3OD (δ 3.31 for ^1H , δ 49.0 for ^{13}C). Infrared spectra were recorded on a NaCl plate using a Perkin-Elmer 1600 series Fourier transform spectrometer. High resolution mass spectra were obtained by Dr. Rakesh Kohli at the University of Pennsylvania Mass Spectrometry Service Center on an Autospec high resolution double-focusing electrospray ionization/chemical ionization spectrometer with either DEC 11/73 or OPUS software data system. All compounds were chromatographically homogeneous materials that were determined to be >95% pure by ^1H and ^{13}C NMR, and where necessary, HPLC.

8. Synthesis of TAK-X-TAK dimers.

To diacid **2–6** (Figure 1)²⁹ (0.225 mmol), TAK aminobenzothiazole **1** (Figure 1)²² (0.472 mmol), and DIPEA (1.3 mmol) in DMF (0.3 M) was added BEP (0.582 mmol). The reaction

was then stirred at 25 °C for 18 h. The reaction was then quenched with brine and extracted with 9:1 ethyl acetate: THF. The combined organic fractions were then washed with brine, dried over Na₂SO₄, filtered and concentrated to afford a crude solid. The crude mixture was purified by silica gel column chromatography (MeOH/DCM) and then purified by preparative thin layer chromatography (MeOH/DCM) to afford TAK-X-TAK dimers as thin films.

TAK-0-TAK

Thin Film; Yield=15%; ¹H NMR (500 MHz, Acetone-*d*₆) δ 9.35 (s, 1H), 8.17 (s, 2H), 7.99 (d, *J*= 9.0 Hz, 2H), 7.73 (s, 1H), 7.68 (d, *J*= 7.2 Hz, 2H), 7.63 – 7.52 (m, 3H), 7.31 – 7.24 (m, 2H), 7.14 (d, *J*= 8.9, 1.6 Hz, 2H), 6.93 (dd, *J*= 9.1, 3.7 Hz, 2H), 4.70 (s, 3H), 3.99 (s, 3H) ppm; ¹³C NMR (126 MHz, CDCl₃) δ 170.03, 169.74, 157.46, 156.34, 151.64, 151.21, 149.30, 149.28, 144.81, 142.46, 139.03, 137.30, 136.30, 133.87, 129.68, 129.64, 129.43, 129.19, 127.97, 127.87, 127.18, 126.28, 126.25, 126.22, 126.20, 125.68, 123.76, 123.73, 123.52, 117.20, 117.11, 116.94, 115.65, 115.59, 114.71, 114.13, 95.83, 70.02, 42.36 ppm; FT-IR (neat) : 1703, 1663, 1625, 1595, 1545 cm⁻¹; HRMS (ESI) *m/z* calcd for C₅₀H₃₀F₈N₈O₇S₂ (M + Na)⁺ 1093.1449; Found 1093.1472.

TAK-2-TAK

Thin Film; Yield = 6%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.21 (s, 1H), 7.93 (d, *J*= 9.0 Hz, 1H), 7.83 (s, 0H), 7.67 (s, 1H), 7.59 (s, 3H), 7.57 – 7.49 (m, 1H), 7.41 – 7.30 (m, 1H), 7.02 (d, *J*= 9.0 Hz, 1H), 6.94 (d, *J*= 8.9 Hz, 1H), 4.34 (s, 2H), 3.88 (s, 2H), 3.79 – 3.72 (m, 3H), 3.69 – 3.62 (m, 3H) ppm; ¹³C NMR (126 MHz, DMSO) δ 170.35, 169.34, 157.14, 155.72, 151.26, 151.24, 150.80, 148.87, 144.42, 136.92, 135.88, 133.48, 129.61, 129.29, 129.26, 129.04, 128.79, 128.54, 127.57, 127.46, 126.60, 125.90, 125.87, 125.84, 125.81, 125.30, 123.40, 123.37, 123.34, 123.31, 123.14, 120.97, 116.70, 116.62, 116.52, 115.20, 115.14, 114.28, 113.75, 113.63, 95.31, 70.38, 69.85, 69.32, 41.97 ppm; FT-IR (neat) : 1675, 1596, 1544, 1485, 1460 cm⁻¹; HRMS (ESI) *m/z* calcd for C₅₄H₃₈F₈N₈O₉S₂ (M + Na)⁺ 1181.1973; Found 1181.1975

TAK-3-TAK

Thin Film; Yield = 8%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.44 (s, 1H), 10.18 (s, 1H), 7.95 (dd, *J*= 9.1, 4.1 Hz, 1H), 7.83 (s, 1H), 7.66 (s, 1H), 7.63 – 7.49 (m, 3H), 7.38 – 7.30 (m, 1H), 7.04 (s, 1H), 6.98 – 6.90 (m, 1H), 4.32 (s, 2H), 3.86 (s, 2H), 3.72 – 3.65 (m, 2H), 3.60 (s, 4H) ppm; ¹³C NMR (126 MHz, DMSO) δ 170.26, 169.38, 157.00, 155.83, 151.25, 150.80, 148.87, 144.43, 136.93, 135.87, 133.52, 129.33, 129.30, 129.26, 129.05, 128.80, 128.55, 127.60, 127.49, 126.75, 126.66, 125.92, 125.83, 125.33, 123.38, 123.16, 116.73, 116.66, 116.57, 115.24, 114.34, 113.80, 113.69, 95.35, 70.52, 69.75, 69.31, 41.98 ppm; FT-IR (neat) : 1690, 1596, 1537, 1458, 1431 cm⁻¹; HRMS (ESI) *m/z* calcd for C₅₆H₄₂F₈N₈O₁₀S₂ (M + H)⁺ 1203.2416; Found 1203.2382.

TAK-4-TAK

Thin Film; Yield = 10%; ¹H NMR (500 MHz, Acetone-*d*₆) δ 9.34 (s, 1H), 8.12 (dd, *J*= 6.6, 3.1 Hz, 1H), 7.86 (d, *J*= 9.0 Hz, 1H), 7.71 (s, 1H), 7.65 (d, *J*= 7.9 Hz, 1H), 7.60 – 7.48 (m,

2H), 7.23 (dd, $J = 10.7, 9.0$ Hz, 1H), 7.03 (d, $J = 9.0$ Hz, 1H), 6.90 (ddd, $J = 9.0, 3.9, 3.1$ Hz, 1H), 4.32 (s, 2H), 3.96 (s, 2H), 3.87 – 3.80 (m, 4H), 3.79 – 3.70 (m, 4H) ppm; ^{13}C NMR (126 MHz, Acetone- d_6) δ 171.48, 170.01, 158.00, 157.26, 152.84, 152.82, 151.44, 149.52, 145.73, 137.75, 137.44, 134.37, 131.22, 130.97, 130.71, 130.46, 130.09, 129.09, 128.99, 127.38, 127.10, 127.06, 127.03, 127.00, 124.47, 124.45, 124.41, 124.38, 124.28, 117.50, 117.08, 116.90, 115.68, 115.62, 114.88, 114.02, 97.17, 72.52, 71.45, 71.11, 70.96, 70.54, 43.56 ppm; FT-IR (neat) : 1684, 1625, 1592, 1538, 11487 cm^{-1} ; HRMS (ESI) m/z calcd for $\text{C}_{58}\text{H}_{46}\text{F}_8\text{N}_8\text{O}_{11}\text{S}_2$ ($\text{M} + \text{Na}$) $^{+}$ 1269.2497; Found 1269.2457.

TAK-6-TAK

Thin Film; Yield = 7%; ^1H NMR (500 MHz, Chloroform- d) δ 10.67 (s, 1H), 8.11 (dd, $J = 6.6, 3.1$ Hz, 1H), 7.78 (d, $J = 9.0$ Hz, 1H), 7.62 – 7.44 (m, 5H), 7.06 (t, $J = 10.4, 8.9$ Hz, 1H), 6.94 (d, $J = 9.0$ Hz, 1H), 6.80 – 6.74 (m, 1H), 4.30 (s, 2H), 3.84 – 3.69 (m, 11H), 3.66 – 3.57 (m, 5H) ppm; ^{13}C NMR (126 MHz, CDCl_3) δ 169.48, 168.09, 159.11, 156.87, 156.62, 151.73, 149.96, 148.04, 144.53, 136.96, 134.64, 132.73, 131.33, 131.07, 129.41, 127.14, 127.05, 126.25, 126.09, 126.06, 124.85, 124.42, 124.40, 116.16, 115.71, 115.55, 115.09, 114.03, 112.80, 96.53, 71.56, 70.68, 70.43, 70.39, 70.36, 70.15, 69.90, 43.98 ppm; FT-IR (neat) : 1693, 1595, 1537, 1485, 1456 cm^{-1} ; HRMS (ESI) m/z calcd for $\text{C}_{62}\text{H}_{54}\text{F}_8\text{N}_8\text{O}_{13}\text{S}_2$ ($\text{M} + \text{H}$) $^{+}$ 1335.3202; Found 1335.3214.

TAK-L

To a solution 2-(2-Methoxyethoxy)acetic acid **12** (Figure 5a; 57 μL , 0.504 mmol), TAK benzotriazole **1**²² (Figure 5a; 0.232 mg, 0.458 mmol), and DIPEA (0.498 mL, 2.8 mmol) in DMF (1.5 mL) was added BEP (0.169 g, 0.620 mmol). The reaction was then stirred at 25 $^{\circ}\text{C}$ for 24 h. The reaction was then quenched with brine and extracted with 9:1 ethyl acetate: THF. The combined organic fractions were then washed with brine, dried over Na_2SO_4 , filtered and concentrated to afford a crude foam. The crude product was purified by silica gel column chromatography (5% MeOH/DCM) and then purified by preparative thin layer chromatography (4% MeOH/DCM) to afford TAK-L **13** (Figure 5a) as a white foam.

Yield = 0.128 g (44% yield); ^1H NMR (500 MHz, Chloroform- d) δ 10.91 (s, 1H), 8.15 (dd, $J = 6.4, 3.1$ Hz, 1H), 7.84 (d, $J = 9.0$ Hz, 1H), 7.62 – 7.48 (m, 4H), 7.48 – 7.41 (m, 1H), 7.08 (t, $J = 10.5, 8.9$ Hz, 1H), 6.97 (d, $J = 9.0$ Hz, 1H), 6.79 (ddd, $J = 8.9, 4.1, 2.9$ Hz, 1H), 4.30 (s, 2H), 3.87 – 3.77 (m, 4H), 3.69 – 3.62 (m, 2H), 3.57 (s, 3H) ppm; ^{13}C NMR (126 MHz, CDCl_3) δ 170.31, 168.88, 168.81, 156.95, 156.41, 151.84, 150.45, 148.53, 144.90, 136.73, 136.50, 133.28, 130.24, 129.99, 129.73, 129.48, 129.04, 128.15, 128.05, 127.96, 127.56, 126.52, 126.00, 125.96, 123.40, 123.36, 116.62, 116.02, 115.85, 114.59, 114.53, 113.83, 113.06, 96.25, 71.46, 71.18, 69.58, 57.95, 42.56 ppm; FT-IR (neat) : 1686, 1533, 1486, 1458, 1429 cm^{-1} ; HRMS (ESI) m/z calcd for $\text{C}_{28}\text{H}_{22}\text{N}_4\text{O}_5\text{F}_4\text{S}$ ($\text{M} + \text{Na}$) $^{+}$ 625.1145; Found 625.144.

2-(2-(2-(2-oxo-2-(thiazol-2-ylamino)ethoxy)ethoxy)ethoxy)acetic acid.

To 3,–6,–9-Trioxaundecandioic acid **3** (2.5 g, 11.25 mmol) in DCM (50 mL) was added DCC (2.32 g, 11.25 mmol). The reaction was then stirred at 25 $^{\circ}\text{C}$ for 18 h. The resulting suspension was then filtered and rinsed with chilled DCM. The filtrate was concentrated to

afford the crude anhydride, which was taken directly on to the next step. The anhydride was dissolved with dry THF and was then treated with 2-aminothiazole (1.12 g, 11.25 mmol). The reaction was stirred for 3 hrs at 25°C and volatiles then removed by evaporation under reduced pressure. The resulting solid was then suspended in Et₂O and filtered. The solid was then rinsed with chilled Et₂O. The crude solid was then purified by silica gel column chromatography (2–10% MeOH/DCM) to give 2-(2-(2-(2-oxo-2-(thiazol-2-ylamino)ethoxy)ethoxy)ethoxy)acetic acid.

Pale yellow solid; Yield = 1.57 g (46% yield); ¹H NMR (500 MHz, Methanol-*d*₄) δ 7.46 (d, *J* = 3.7 Hz, 1H), 7.14 (d, *J* = 3.7 Hz, 1H), 4.29 (s, 2H), 4.11 (d, *J* = 3.4 Hz, 2H), 3.81 – 3.71 (m, 8H); ¹³C NMR (126 MHz, DMSO) δ 171.68, 168.50, 157.37, 137.65, 113.68, 70.49, 69.81, 69.75, 69.60, 69.24, 67.59, 40.02, 39.86, 39.69, 39.52, 39.35, 39.19, 39.02; IR (neat) : 3200, 1719, 1689, 1495, 1063 cm⁻¹; HRMS (ESI) *m/z* calcd for C₄₈H₄₁N₆O₈F₄S₂ (M + H)⁺ 305.0822; Found 305.0807.

TAK-L-C

To a solution of 2-(2-(2-(2-oxo-2-(thiazol-2-ylamino)ethoxy)ethoxy)ethoxy)acetic acid (0.159 mg, 0.524 mmol), TAK aminobenzothiazole²² (0.085 mg, 0.174 mmol) and TEA (0.243 ml, 1.74 mmol) in DMF (0.587 mL) was added T3P (0.333 mL, 0.524 mmol) in DMF (1:1; v/v) dropwise. The reaction was then stirred at 25 °C for 18 h. The reaction was then quenched with brine and extracted with 9:1 ethyl acetate: THF. The combined organic fractions were then washed with brine, dried over Na₂SO₄, filtered and concentrated to afford a crude solid. The crude product was then purified by preparative thin layer chromatography (3% MeOH/chloroform) to afford TAK-control-2 as a thin film.

Thin Film; Yield = 0.135g (55% yield); ¹H NMR (500 MHz, Chloroform-*d*) δ 10.55 (s, 1H), 10.35 (s, 1H), 8.11 (dd, *J* = 6.5, 3.1 Hz, 1H), 7.72 (d, *J* = 9.0 Hz, 1H), 7.62 (d, *J* = 3.4 Hz, 1H), 7.60 – 7.45 (m, 4H), 7.37 (d, *J* = 3.7 Hz, 1H), 7.06 (t, *J* = 9.7 Hz, 1H), 6.95 – 6.86 (m, 2H), 6.76 (dt, *J* = 8.9, 3.5 Hz, 1H), 4.31 (s, 2H), 4.24 (s, 2H), 4.06 – 3.99 (m, 4H), 3.85 (dt, *J* = 4.2, 2.3 Hz, 4H), 3.80 (s, 2H) ppm; ¹³C NMR (126 MHz, CDCl₃) δ 169.38, 168.05, 157.07, 156.82, 156.63, 151.76, 149.96, 148.04, 144.29, 137.57, 137.38, 136.91, 136.46, 134.69, 132.74, 131.31, 131.05, 130.80, 129.39, 127.16, 127.07, 126.11, 126.09, 126.06, 126.03, 124.87, 124.41, 124.38, 122.71, 116.19, 115.68, 115.51, 115.09, 115.02, 114.03, 113.76, 112.88, 96.47, 71.41, 71.33, 70.42, 69.77, 69.74, 43.97 ppm; FT-IR (neat) : 1692, 1595, 1533, 1484, 1455 cm⁻¹; HRMS (ESI) *m/z* calcd for C₃₄H₂₈N₆O₇F₄S₂ (M + H)⁺ 773.1475; Found 773.1464.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations Used

MAPK	mitogen-activated protein kinase
BSA	bovine serum albumin
TBST	Tris-buffered saline with Tween-20
MEK	mitogen-activated protein kinase kinase
RTKs	receptor-tyrosine kinases
FDA	Food and Drug Administration
ELISA	Enzyme-linked immunosorbent assay
GST	glutathione S-transferase
AUC	Analytical Ultracentrifuge
DSC	Differential Scanning Calorimetry
PDB	Protein Data Bank
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HPLC	high-pressure liquid chromatography
TLC	thin layer chromatography
DIPEA	diisopropylethylamine
BEP	2-Bromo-1-ethylpyridinium tetrafluoroborate
DCM	dichloromethane
DMF	dimethylformamide
THF	tetrahydrofuran
DCC	dicyclohexylcarbodiimide
TEA	trimethylamine
T3P	propylphosphonic anhydride

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Figure 1. Structure of TAK632 bound BRAF and rationale for linked TAK inhibitors.

(a) Structure of BRAF^{WT}/TAK632 (accession code 4KSP), highlighting the exposed cyclopropane ring, (b) Structure of monovalent TAK632, and where it interacts with residue Cys532 of BRAF (c) Initial scaffold for bivalent TAK inhibitors where n equals the number of ethylene glycol moiety groups present, (d) Alignment of BRAF^{WT}/TAK632 (pink and grey) with BRAF^{V600E}/Vem-BisAmide-2 structure (blue) (5JT2). (e). Preparation of bivalent TAK-n-TAK structures

Figure 2. Potency of first generation bivalent TAK inhibitors.

(a) Dose response curves of bivalent TAK inhibitors against BRAF^{WT} with TAK632 as a control. Calculated IC₅₀ values are indicated. The experiments were performed in triplicate with $\pm$ SEM shown. 95% confidence intervals are: TAK632 (1.47 nM to 7.47 nM), TAK-2-TAK (69.6 nM to 249 nM), TAK-4-TAK (48.4 nM to 168 nM), TAK-O-TAK (347 nM to 832 nM), TAK-3-TAK (351 nM to 1.08 μ M), and TAK-6-TAK (277 nM to 1.94 μ M).

(b) Dose response curves of bivalent TAK inhibitors against BRAF^{V600E} with TAK632 as a control, carried out in triplicate with $\pm$ SEM shown. The 95% confidence intervals are: TAK632 (2.67 nM to 7.45 nM), TAK-2-TAK (47.5 nM to 115 nM), TAK-4-TAK (59.7 nM to 91.1 nM), TAK-O-TAK (323 nM to 549 nM), TAK-3-TAK (128 nM to 219 nM), and TAK-6-TAK (199 nM to 621 nM).

Figure 3. Sedimentation velocity experiments of bivalent TAK Inhibitors.

(a) BRAFR^{R509H} (10 μ M) in the absence and presence of TAK632 and TAK-2-TAK inhibitor at different concentrations, (b) BRAFR^{R509H} (12 μ M) in the absence and presence of TAK632 and TAK-4-TAK inhibitor at different concentrations, (c) BRAFR^{R509H} (12 μ M) in the absence and presence of TAK632, TAK-3-TAK and TAK-6-TAK inhibitors at 20 μ M.

Figure 4. Sedimentation velocity and sedimentation equilibrium experiments of bivalent TAK Inhibitors.

(a) BRAF^{R509H} (20 μM) in the absence and presence of TAK632 and TAK-4-TAK inhibitor at different concentrations, (b) BRAF^{WT} (10 μM) in the absence and presence of TAK632 and TAK-2-TAK. (c) BRAF^{V600E} (10 μM) in the absence and presence of TAK632 and TAK-2-TAK and TAK-4-TAK inhibitors at different concentrations, (d) Log plots of sedimentation equilibrium experiments showing theoretical monomer (purple) and theoretical dimer (blue) slopes of BRAF compared to BRAF^{R509H} in the absence and presence of TAK632 and bivalent TAK-4-TAK at a 2:1 molar ratio of inhibitor to protein.

Figure 5. Biochemical and biophysical properties of TAK-linker compounds.

(a) Preparation of monomeric TAK control compounds, TAK-L (TAK with linker) and TAK-L-C (TAK with linker and cap), (b). Dose response curves of TAK control compounds along with bivalent TAK-2-TAK and TAK-4-TAK and TAK632 against BRAF^{WT}. Calculated IC₅₀ values are indicated. The experiments are performed in duplicate with +/- SEM shown. 95% confidence intervals are: TAK-L (3.33 nM to 9.91 nM), TAK-L-C (10.3 nM to 40.2 nM), TAK-2-TAK (50.2 nM to 151 nM), TAK-4-TAK (56.7 nM to 129 nM), and TAK632 (3.32 nM to 15.2 nM). (c). Sedimentation velocity experiments with BRAF^{R509H} (15 μM) in the presence of TAK-L, TAK-L-C, and TAK-2-TAK at 20 μM.

Figure 6. Determination of bivalent TAK inhibitor binding modes to BRAF

(a) Limited proteolysis experiment of BRAF^{WT} in the presence and absence of 200 μM inhibitors after exposure to trypsin for 30 minutes (lanes 1–5) and lane 6 shows BRAF^{WT} without ligand or trypsin, (b) Differential Scanning Calorimetry (DSC) experiments in which BRAF^{WT} is heated in the presence and absence of inhibitors ranging in concentration from 25 μM to 250 μM. Melting temperatures are as follows: 125 μM TAK632–68.74 °C; 125 μM TAK-L-C–63.29 °C; 75 μM TAK-2-TAK–38.37 °C, 58.65 °C; 125 μM TAK-2-TAK–38.72 °C, 59.6 °C; 250 μM TAK-2-TAK–40.08 °C, 62.64 °C; No ligand–37.84 °C.

Figure 7. BRAF^{R509H} dimer interface via TAK632 binding

(a) Overlay of BRAF^{WT}/TAK632 (light pink and magenta), BRAF^{V600E}/AZ628 (light grey and dark grey), BRAFR509H/AZ628 (blue and teal), and BRAFv600E/Vemurafenib (yellow) structures, highlighting the shift within the αC-in position. Different subunits of the crystal structures are denoted with subscript 1 and 2, respectively, (b) Overlay of BRAF^{WT}/TAK632 (light pink, PDB ID: 4KSP), BRAF^{V600E}/AZ628 (grey, PDB ID: 4G9R), and BRAFR509H/AZ628 (blue, PDB ID: 4RZW) highlighting a shift in the W450 residue in the BRAFR509H structure, (c) Crystal structure of BRAF^{R509H} bound to AZ628 demonstrates that W450 forms pi-stacking interactions with His509. (d) Residue D448 accompanies the shift in the W450 residue, hydrogen bonding with the other static W450. (e) R506 residues

in both subunits of BRAF^{wt}/TAK632 (light pink and magenta), BRAF^{V600E}/AZ628 (light grey and dark grey), and BRAF^{R509H}/AZ628 (blue and teal) aligned with one subunit of BRAF^{V600E}/Vemurafenib (yellow) demonstrating “in” and “out” positions of R506. (f). W450 lies within close proximity of R506, and the W450 in BRAF^{R509H}/AZ628 shifts closer to R506.

Figure 8. Sedimentation velocity experiments of dimerization mutants and aC-in/aC-out inhibitors.

(a) Sedimentation velocity experiments of BRAF^{R509H}, BRAF^{R506A}, and BRAF^{W450A} in the absence and presence of TAK632 at 15–25 μ M. (b) Sedimentation velocity experiments of BRAF^{R509H}, BRAF^{R506A/R509H}, BRAF^{W450A/R509H} and BRAF^{R506A/W450A/R509H} in the absence and presence of TAK at 25 μ M. (c) Sedimentation velocity experiments of BRAF^{R509H} without inhibitor and with 20 μ M of both TAK632 and sorafenib. (d) Sedimentation velocity experiments of BRAF^{WT} and BRAF^{V600E} with 15 μ M vemurafenib.

Figure 9. Superposition of BRAF^{V600K}/Vem-BisAmide-2 with BRAF^{WT}/TAK632.

(a) Overlay of BRAF^{WT} co-crystallized with TAK632 (PDB ID: 4KSP) overlaid with the aC-out/aC-out “face-to-face” dimer induced by Vem-BisAmide-2 (PDB ID: 5JT2). (b) Comparison of activation segment of BRAF^{V600E} bound to Vem-BisAmide-2 in an active conformation (cyan) and the activation segment of BRAF^{WT} bound to TAK632 in an inactive conformation (magenta), protruding into the other molecule of the “face-to-face” dimer.