

Mechanism of Dimer Selectivity and Binding Cooperativity of BRAF Inhibitors

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eLife Assessment

This **fundamental** study illuminates the dynamics of BRAF in its monomeric and dimeric forms, both in the absence and presence of inhibitors, through a **convincing** combination of traditional experiments and sophisticated computational analyses. By revealing novel insights into the selectivity and cooperative processes of BRAF inhibitors, it holds significant promise for the development of future therapeutics, particularly against mutant isoforms in cancer. Overall, these findings will be of great interest to structural biologists, medicinal chemists, and pharmacologists.

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Abstract

Aberrant signaling of BRAF^{V600E} is a major cancer driver. Current FDA-approved RAF inhibitors selectively inhibit the monomeric BRAF^{V600E} and suffer from tumor resistance. Recently, dimer-selective and equipotent RAF inhibitors have been developed; however, the mechanism of dimer selectivity is poorly understood. Here, we report extensive molecular dynamics (MD) simulations of the monomeric and dimeric BRAF^{V600E} in the apo form or in complex with one or two dimer-selective (PHI1) or equipotent (LY3009120) inhibitor(s). The simulations uncovered the unprecedented details of the remarkable allostery in BRAF^{V600E} dimerization and inhibitor binding. Specifically, dimerization retrains and shifts the α C helix inward and increases the flexibility of the DFG motif; dimer compatibility is due to the promotion of the α C-in conformation, which is stabilized by a hydrogen bond formation between the inhibitor and the α C Glu501. A more stable hydrogen bond further restrains and shifts the α C helix inward, which incurs a larger entropic penalty that disfavors monomer binding. This mechanism led us to propose an empirical way based on the co-crystal structure to assess the dimer selectivity of a BRAF^{V600E} inhibitor. Simulations also revealed that the positive cooperativity of PHI1 is due to its ability to preorganize the α C and DFG

conformation in the opposite protomer, priming it for binding the second inhibitor. The atomically detailed view of the interplay between BRAF dimerization and inhibitor allostery as well as cooperativity has implications for understanding kinase signaling and contributes to the design of protomer selective RAF inhibitors.

Introduction

The mitogen activated protein kinase (MAPK) signaling cascades regulate cell growth, proliferation, and survival in mammalian cells (Samatar and Poulikakos, 2014; Lavoie et al., 2020). In the well-studied Ras-Raf-MEK-ERK pathway, the GTP-loaded RAS contacts RAF and induces its dimerization; the newly formed RAF dimer phosphorylates MEK which in turn phosphorylates ERK, which then phosphorylates a number of downstream proteins and regulate their functions (Lavoie et al., 2020). Mutations of BRAF, a kinase within the RAF family, are present in about 8% of human tumors, most commonly melanoma and colorectal cancers, with the mutation V600E accounting for about 90% of them. It is believed that the wild type BRAF signals as a dimer, the BRAF^{V600E} is able to signal as a monomer (Poulikakos et al., 2010, 2011; Karoulia et al., 2017). The first generation BRAF^{V600E} inhibitors, including the current FDA-approved inhibitors, Vemurafenib, Dabrafenib, and Encorafenib, inhibit the monomeric BRAF^{V600E}; however, drug resistance led to only short-term cancer remission in patients (Poulikakos et al., 2011; Peng et al., 2015; Monaco et al., 2021; Yen et al., 2021; Adamopoulos et al., 2021). In the adaptive drug resistance mechanism, RAF dimerization renders the monomer-selective inhibitors ineffective (Poulikakos et al., 2011; Peng et al., 2015; Monaco et al., 2021; Yen et al., 2021; Adamopoulos et al., 2021). To overcome the resistance, inhibitors that are either dimer selective or equipotent to both monomers and dimers have been developed and entered clinical development (Adamopoulos et al., 2021; Cook and Cook, 2021). Understanding the molecular mechanism of dimer selectivity would be valuable for the rational design of RAF inhibitors. Although several MD studies have examined the conformational dynamics of BRAF^{V600E} monomer (Maloney et al., 2021), BRAF^{V600E} in complex with the monomer-selective inhibitors (Tse and Verkhivker, 2016), and wild type RAF dimerization (Zhang et al., 2021), the topic of RAF dimer selectivity has not been explored.

The kinase domain of the BRAF monomer has a typical kinase structure: a primarily β -sheet N-terminal domain connected to a helical C-terminal domain by a flexible hinge (Figure 1). Like other kinases, the catalytic activity of BRAF depends on the conformation of two motifs: the α C-helix, which contains the conserved residue Glu501, and the DFG motif on the activation loop (α -loop), which contains the conserved ATP-binding (via magnesium) residue Asp594. In the active state, both the α C helix and DFG adopt the IN conformation, dubbed CIDI. In this state, the α C helix is positioned inward such that α C-Glu501 and the catalytic Lys483 form a salt bridge; meanwhile the DFG motif is also IN, meaning DFG-Asp594 is near Lys483 often in a salt-bridge distance. An inactive conformation can be achieved if either or both the α C helix and DFG motif adopt an OUT state. Specifically, α C-out involves an outward movement of the α C helix, while DFG-out involves the sidechains of the DFG Asp594 and Phe595 exchanging regions, i.e., Phe595 facing the ATP binding site and Asp594 facing the α C-helix. In the BRAF dimer, the two protomers are arranged side by side and the dimer interface involves the C-terminal end of the α C helix (Figure 1). Current monomer-selective BRAF^{V600E} inhibitors bind in the α C-out conformation, whereas the dimerselective or equipotent inhibitors bind in the α C-in conformation (Supplemental Table 1). Thus, the α C conformation has been the center of attention in numerous structural and biochemical studies to understand RAF signaling and inhibitor activities (Rajakulendran et al., 2009; Thevakumaran et al., 2015; Karoulia et al., 2016).

In a recent study, Gavathiotis and coworkers discovered a modification to the dimer-compatible inhibitor Ponatinib which can increase the dimer selectivity by more than three fold (Cotto-Rios et al., 2020). The novel inhibitor, named Ponatinib hybrid inhibitor 1 (PHI1), extends the head-

Table 1.

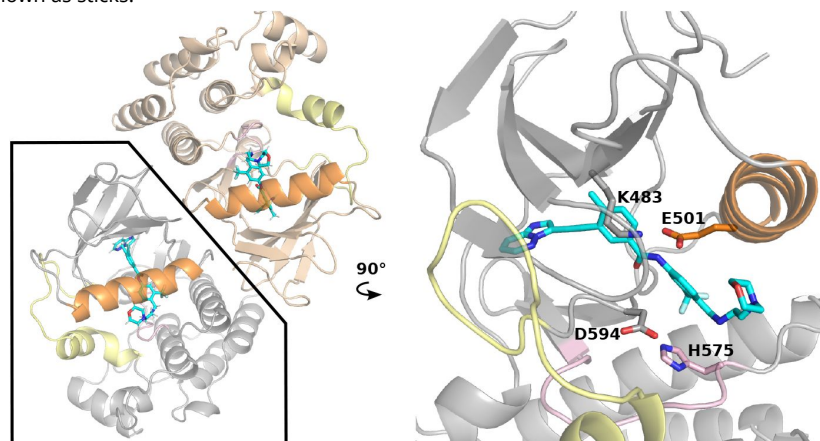
Summary of the fixed-protonation-state MD simulations (aggregate time of 135 μ s)

No.	System	Simulation time	Starting structure
1	Apo monomer	3 x 5 μ s	6P7G(A)
2	Apo dimer	3 x 5 μ s	6P7G (inhibitors removed)
3	Holo monomer:PHI1	6 x 5 μ s	6P7G (A:PHI1 or B:PHI1)
4	Holo monomer:LY	3 x 5 μ s	5C9C (A:LY)
5	Mixed dimer:PHI1	3 x 5 μ s	6P7G (apo A; B:PHI1)
6	Holo dimer:2PHI1	3 x 5 μ s	6P7G (A:PHI1, B:PHI1)
7	Mixed dimer:LY	3 x 5 μ s	5C9C (A:LY; apo B)
8	Holo dimer:2LY	3 x 5 μ s	5C9C (A:LY; B:LY)

Figure 1.

The X-ray structure of the BRAFV600E dimer in complex with PHI1.

Left. Cartoon representation of the BRAFV600E dimer in complex with PHI1 (PDB: 6P7G *Cotto-Rios et al. (2020)*, two protomers are colored tan and grey). The α C-helix, a-loop, and c-loop are colored orange, yellow, and pink, respectively. **Right.** A zoomed-in view of a PHI1-bound protomer. PHI1 and the sidechains of DFG-Asp594, α C-Glu501, catalytic Lys483, and HRD-His574 are shown as sticks.



group of Ponatinib by replacing the methylpiperazine with the 4-(2-aminoethyl) morpholino group. Remarkably, PHI1 showed more potent inhibition of the second protomer in the BRAF^{V600E} dimer; in contrast, Ponatinib and equipotent inhibitors, e.g., LY3009120 or LY, AZ-628, and TAK-632, are non-cooperative (Cotto-Rios et al., 2020). The co-crystal structure of BRAF^{V600E} in complex with PHI1 (PDB: 6P7G) (Cotto-Rios et al., 2020) revealed that the morpholine group extends the ligand-kinase interaction from the type-II pocket (occupied by all DFG-out inhibitors) to the center of α C helix, allowing a hydrophobic interaction with Asn500 next to the α C-Glu501 (Figure 1). The co-crystal structures show that this interaction is not available with the shorter Ponatinib (PDB ID: 6P3D) (Cotto-Rios et al., 2020) or equipotent inhibitors, e.g., LY3009120 (LY, PDB ID: 5C9C) (Peng et al., 2015). Gavathiothis and coworkers noticed that PHI1 stabilizes the α C helix in a slightly different IN conformation as compared to Ponatinib and hypothesized that the additional interaction with Asn500 is a key to the dimer selectivity of PHI1, as it may be unfavorable in monomer binding (Cotto-Rios et al., 2020). Shortly after, a biochemical study supported by the molecular dynamics (MD) simulations suggested that restriction of the α C helix movement is the basis for the difference between dimer-selective and equipotent inhibitors (Adamopoulos et al., 2021); however, the detailed mechanism remains elusive.

Prompted by the open questions regarding dimer selectivity and binding cooperativity of BRAF^{V600E} inhibitors, we carried out a series of all-atom molecular dynamics (MD) simulations to investigate the conformational dynamics of the monomeric and dimeric BRAF^{V600E} in the presence and absence of one or two dimer-selective (PHI1) or equipotent (LY) inhibitor(s). Analysis of the simulation data which amounts to 135 μ s aggregate time uncovered the atomic details of the remarkable conformational allostery in BRAF^{V600E} dimerization and inhibitor binding. Supported by the co-crystal structure analysis of the published monomer-selective, dimer-selective, and equipotent inhibitors, an atomically-detailed mechanism emerged that explains the monomer or dimer selectivity and binding cooperativity of BRAF^{V600E} inhibitors. The mechanism also led us to propose an empirical method based on the co-crystal structure for assessing the dimer selectivity of BRAF^{V600E} inhibitors.

Results and Discussion

Analysis of the co-crystal structures suggests the h-bond formation with α C-Glu501 as a key requirement for dimer binding

To understand the preference of BRAF^{V600E} inhibitors for the monomer vs. dimer form, we first examined all published co-crystal structures in complex with the monomer-selective and dimer-compatible (i.e., dimer-selective and equipotent) inhibitors (see Supplementary table 1 for a complete list). We first noticed that the monomer-selective inhibitors, e.g., Vemurafenib (VEM, PDB ID: 5JRQ) (Grasso et al., 2016), do not occupy BP-III, whereas most dimer-compatible inhibitors do. This can be explained by the observation that the monomer-selective inhibitors bind in the DFG-in, whereas most dimer-compatible inhibitors bind in the DFG-out conformation—BP-III is occupied by Phe595 in the DFG-in conformation, so the pocket is only available in the DFG-out conformation (Figure 2a). Note, the equipotent inhibitor SB590885 (PDB ID: 2FB8) (King et al., 2006) does not occupy BP-II or BP-III, as it binds in the DFG-in conformation (Supplementary table 1).

The co-crystal structure analysis revealed an important distinction between the monomer-selective and dimer-compatible inhibitors, namely, the former binds in the α C-out whereas the latter binds in the α C-in conformation. The interaction fingerprints showed that while most monomer-selective inhibitors make a hydrophobic contact with Leu505 next to the conserved RKTR motif at the end of the α C helix, only the dimer-compatible inhibitors interact with α C-Glu501 by donating a h-bond (e.g., from an amide group in PHI1 and LY) to the carboxylate sidechain of Glu501 (Figure 2b). Glu501 rests above BP-II in the DFG-out conformation (called

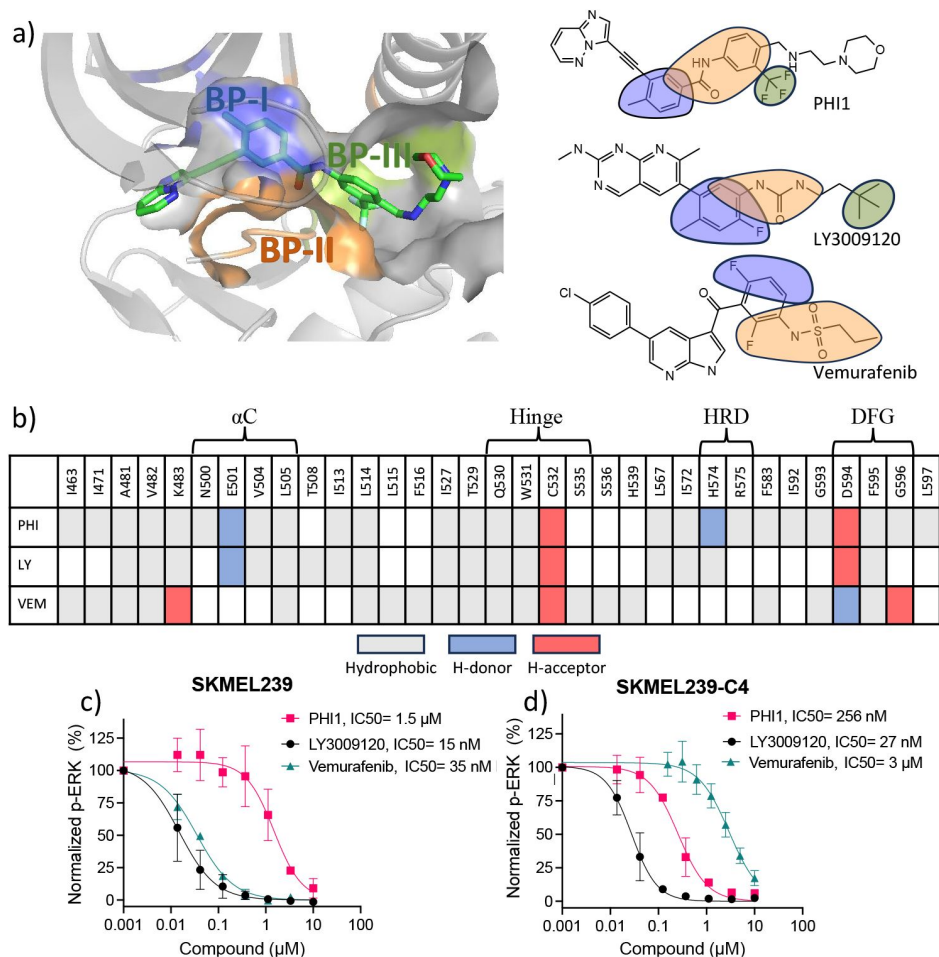


Figure 2.

Protein-ligand interaction fingerprints for PHI1, LY3009120, Vemurafenib, and the inhibition of ERK1/2 phosphorylation in melanoma cells.

a) Left. Visualization of the back pockets (BPs) in BRAFV600E in complex with PHI1. BP-I, BP-II, and BP-III are colored blue, orange, and green, respectively. BP definitions of Liao (Liao, 2007) are followed. **a) Right.** Chemical structures of the example dimer selective (PHI1), equipotent (LY3009120 or LY), and monomer selective (Vemurafenib or VEM) inhibitors of BRAFV600E. Portions of structures are highlighted according to the BPs they occupy in the co-crystal structure (PDB IDs: 6P7G, 5C9C, and 4RZV). **b)** Protein-ligand interaction fingerprints for PHI1, LY, and VEM in BRAFV600E according to the co-crystal structures (PDB IDs: 6P7G, 5C9C, and 4RZV). White indicates no interaction, while grey, blue, and red indicate hydrophobic, h-bond donor (H-donor) and acceptor (H-acceptor) interactions, respectively. These interactions were calculated by KLIFS (Kooistra et al., 2016) and manually verified and corrected. A h-bond was defined using the donor-accept distance cutoff of 3.5 Å, and a hydrophobic contact cutoff of 4 Å was used for aromatic interactions and 4.5 Å for non-aromatic interactions. For simplicity, aromatic face-to-face interactions are indicated as hydrophobic. An extensive list of monomer-selective and dimer-compatible inhibitors with co-crystal structures is given in Supplemental Table 1. **c,d)** Inhibition of ERK1/2 T202/Y204 phosphorylation in SKMEL239 (c) and SKMEL239-C4 (d) melanoma cells (50,000 cells/well) following one hour treatment at 37°C by PHI1, LY3009120, and Vemurafenib in different concentrations. Normalized values and non-linear regression fits of ERK phosphorylation % are shown for different compounds. Error bars represent mean±SEM with n=3.

BP-II-out) and may interact with the catalytic Lys483 (see later discussion), which makes up a part of BP-I. Interestingly, even though SB590885 binds in the DFG-in conformation, it can also donate a h-bond to Glu501 through an oxime hydroxyl group (PDB ID: 2FB8) (King et al., 2006 [\[4\]](#)). This h-bond stabilizes the salt-bridge between the catalytic Lys483 and Glu501 such that the α C helix position is further inward (according to the KLIFS definition, see later discussion) as compared to the co-crystal structures in complex with other dimer-compatible inhibitors (Supplementary Table 1).

All monomer-selective and dimer-compatible inhibitors interact with the DFG-Asp594 although with subtle differences. In PHI1 (PDB ID: 6P7G) and LY (PDB ID: 5C9C), the amide carbonyl occupying the BP-II accepts a h-bond from the backbone amide of Asp594, while in VEM (PDB ID: 4RZV) (Grasso et al., 2016 [\[4\]](#)) the sulfonamide occupying the BP-II donates a h-bond to the backbone amide of Asp594 (orange in **Figure 2a** [\[4\]](#)). One unique property of PHI1 is the ability to donate a h-bond to the backbone carbonyl of HRD-His574 through an amino nitrogen next to the morpholine head-group. This region is classified as BP-IV by KLIFS, although the sidechain of His574 is a part of BP-II (in PDB 6P7G) and makes a h-bond with the backbone of the DFG-1 Gly593. Among the other dimer-compatible inhibitors, only Ponatinib (PDB ID: 6P3D) (Cotto-Rios et al., 2020 [\[4\]](#); Adamopoulos et al., 2021 [\[4\]](#)) makes a similar h-bond with the backbone of His574 through the methyl pyrazine headgroup.

In addition to analyzing the co-crystal structures, we also tested the inhibition of ERK1/2 phosphorylation in two melanoma cell lines by PHI1, LY, or VEM (**Figure 2c,d** [\[4\]](#)). SKMEL239 expresses monomeric BRAF^{V600E}, while SKMEL239-C4 expresses dimeric BRAF^{V600E} (Cotto-Rios et al., 2020 [\[4\]](#)). Among the three inhibitors tested, PHI1 is the only compound to be more potent against SKMEL239-C4 versus SKMEL239 (IC₅₀ of 256 nM vs. 1.5 μ M). By contrast, LY has similar potency (27 nM vs. 15 nM) while VEM is more potent against SKMEL239 (3 μ M vs 35 nM). This data confirms that PHI1 is dimer-selective, LY equipotent, and VEM monomer-selective.

In light of the above finding and given the central location of Glu501 on the α C helix, we hypothesized that the ability to form a h-bond with Glu501 is required by dimer-compatible inhibitors, as the h-bonding would restrict the α C helix to the α C-in conformation as observed in the co-crystal structures of all dimer-compatible inhibitors. This restriction was also suggested as a key for dimer selectivity in the recent study by Poulikakos and coworkers (Adamopoulos et al., 2021 [\[4\]](#)). However, the crystal structures do not provide an explanation for why the ability to induce the α C-in conformation enables the inhibitor to favor dimeric BRAF^{V600E} over monomeric BRAF^{V600E}. Thus, to test the hypothesis regarding the role of h-bond with the Glu501 and to dissect the mechanism of dimer selectivity, we conducted a series of MD simulations of the monomeric and dimeric BRAF^{V600E} in the absence and presence of two dimer-compatible inhibitors (see below).

Overview of the MD simulations of the monomeric and dimeric BRAF^{V600E}

The dimer interface of BRAF^{V600E} contains two histidines, His477 and His510. His510 forms a h-bond with His477 of the opposite protomer, while His477 is also in a potential salt bridge distance from Asp595 of the opposite protomer (Supplemental Figure 2). In a preliminary simulation, where all histidines were set to be neutral and in a tautomer state determined by inspection of the X-ray structure (His477 was set to HID; all others set to the AMBER (Case et al., 2020 [\[4\]](#)) default HIE), we found the BRAF^{V600E} dimer dissociated within a few hundred nanoseconds. To rigorously determine protonation states, we applied the all-atom continuous constant pH molecular dynamics (CpHMD) titration (Harris et al., 2022 [\[4\]](#)), which revealed that His477 is most likely in the charged HIP state while His510 is most likely in the neutral HIE state at neutral pH (Supplemental Figure 3,4 [\[4\]](#)).

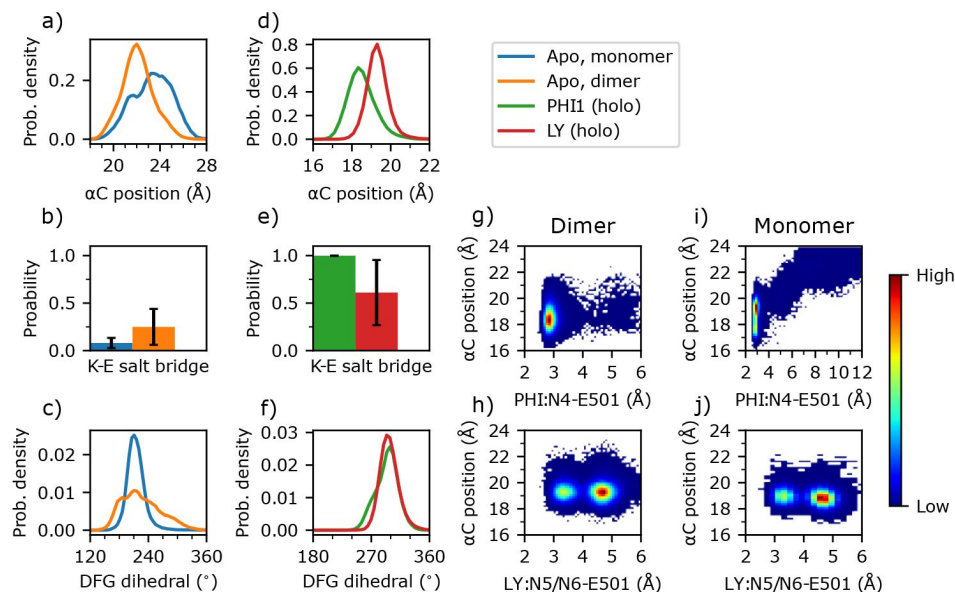


Figure 3.

Dimerization and inhibitor binding modulate the conformation and dynamics of the α C-helix and DFG motif of BRAFV600E.

a-f) Probability distribution of the α C position, probability of the Lys483–Glu501 salt bridge, and probability distribution of the DFG pseudo dihedral angle in the apo monomer (blue), apo dimer (orange), PHI1-bound dimer (green), and LY-bound dimer BRAFV600E (red). The α C position is defined by the distance between the $C\alpha$ of Ile582 on $\beta 7$ and the $C\alpha$ center of mass of Asn500, Glu501, and Val502 (Kanev et al., 2020). A salt bridge between Lys483 and Glu501 is defined by a cutoff distance of 4 $\text{\AA}$ between the nitrogen of Lys483 and the nearest carboxylate oxygen of Glu501; the standard deviation of the probability across replicas are shown as error bars. The DFG pseudo dihedral is defined by the $C\alpha$ atoms of Ile592, Gly593, Asp594, and Phe595 (Möbitz, 2015). **g-j)** Density plots of the α C position vs. the minimum distance between Glu501 and the amide group of PHI1 (g,i) or LY (h,j) in the holo dimer (g,h) or holo monomer (i,j) BRAFV600E.

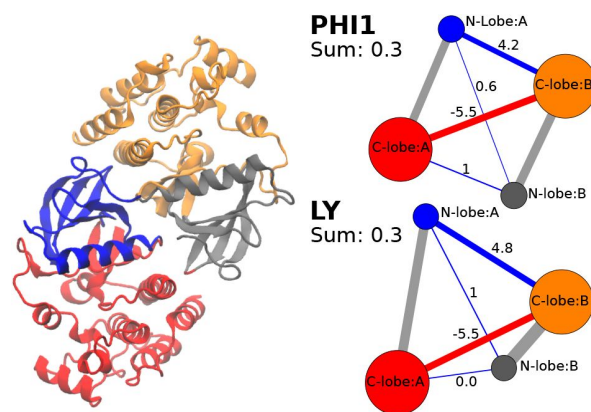


Figure 4.

Both PHI1 and LY stabilize the interprotomer contacts of BRAFV600E.

Left. The N-lobe (blue for A; grey for B) and C-lobe (red for A; orange for B) of each protomer in the BRAFV600E dimer are separated into different communities according to the difference contact network analysis (Yao et al., 2018). **Right.** The average number of interprotomer contacts was calculated for the apo and holo BRAFV600E dimer. (PHI1 top or LY(bottom)). The difference between the holo and apo contacts is shown in the graph form for PHI1 (top) and LY (bottom), and the sum (0.3) is given. Interprotomer contacts are shown as blue (more contacts in holo simulations) or red (more contacts in apo simulations) edges. The difference contact network analysis was performed using the dCNA program (Yao et al., 2018). The cutoff distance defining a contact was 4.5 Å; the threshold for determining a stable contact was set to 0.7, and the number of communities was set to 4.

Based on the CpHMD determined protonation states, we carried out a series of fixed-charge MD simulations of the monomeric and dimeric BRAF^{V600E} in the ligand-free state (apo) or in complex with the PHI1 or LY inhibitor in each protomer (holo). To investigate the cooperativity of inhibitor binding, MD simulations were also conducted where only one protomer is complexed with the PHI1 or LY inhibitor (mixed). Each simulation lasted 5 μ s and was repeated three times for statistical significance; in total, 135 μ s trajectory data was collected (**Table 1**) and the last 3 μ s of each repeat was used for analysis.

We note that enhanced sampling methods were not used due to several challenges. First, the BRAF dimer is weakly associated, with α C helix forming a part of the dimer interface (**Figure 1a**). Enhanced sampling (particularly of α C helix) would likely lead to dimer dissociation. Second, biased sampling methods such as metadynamics may lead to unrealistic conformational states due to the slow relaxation of some parts of the protein to accommodate the conformational change directed by the reaction coordinate. For example, our unpublished metadynamics simulations of a monomer kinase showed that enhancing the DFG conformational change resulted in distortion of the kinase structure.

Dimerization restrains and shifts α C inward while increasing the flexibility of DFG

In order to understand why an inhibitor prefers binding with a dimer or monomer BRAF^{V600E}, it is important to understand the difference in the conformation and dynamics between the apo monomeric and dimeric BRAF^{V600E}. We focus on the α C helix and DFG motif due to their flexibility and importantly specific interactions with the inhibitors (**Figure 2**). Following KLIFS (Kanev et al., 2020), the α C position is characterized by the distance between Ile582 on β 7 (representing a stable reference point) and the center of mass of the α C atoms of Asn500, Glu501, and Val502 (representing the center of the α C helix); a distance below 19.6 Å defines the α C-in while a distance above defines the α C-out states. We also examined the salt-bridge formation between the α C-Glu501 and catalytic Lys483; a minimum sidechain distance below 4.5 Å is an alternative way to define the α C-in states (Tsai et al., 2019; Sultan et al., 2018). These two definitions are consistent and offers complementary information (see later discussion). The holo PHI1-bound structure (PDB: 6P7G) has both protomers resolved with the α C positions of 19.1 and 19.0 Å, suggesting that the α C helix is in but close to the boundary (19.6 Å) with α C-out according to the KLIFS definition (Kanev et al., 2020).

Unlike in the co-crystal structures of dimer-compatible inhibitors, the simulations of the apo monomer and dimer revealed that the α C helix mostly samples the α C-out state. Compared to the apo monomer, the α C position is not only more restrained but also shifted inward by about 1 Å in the apo dimer, as seen from the increase of the peak height and the left-shift of the peak position in the probability distributions, from 23.2 to 22.0 Å (**Figure 3a**, Supplemental Figure 5). The flexibility of the α C position in the apo BRAF^{V600E} is consistent with a previous MD study (Maloney et al., 2021). Enabled by the α C inward movement, the probability of salt-bridge formation between Glu501 and Lys483 is increased by two-fold in the apo dimer (~25%) relative to the apo monomer (~12%, **Figure 3b**, Supplementary Table 3). The enhanced but nonetheless unstable Glu501–Lys483 salt bridge indicates that dimerization primes the α C-helix for adopting the α C-in state, e.g., upon interacting with a dimer-compatible inhibitor.

In contrast to the α C helix, the motion of the DFG motif is significantly enhanced, as evident from the significant widening of the probability distribution of the DFG pseudo dihedral (**Figure 3c**, Supplemental Figure 5), defined by the α C atoms of Ile592 (DFG-2), Gly593 (DFG-2), Asp594 (DFG-Asp), and Phe595 (DFG-Phe) (Möbitz, 2015). Based on a cutoff of 140°, the DFG pseudo dihedral has been found to discriminate between the DFG-in and DFG-out states of kinases (Möbitz, 2015; Tsai et al., 2019). Accordingly, the distributions indicate that the DFG motif samples the DFG-out state in both apo monomer and dimer, with the DFG pseudo dihedral of ~210°; however, the

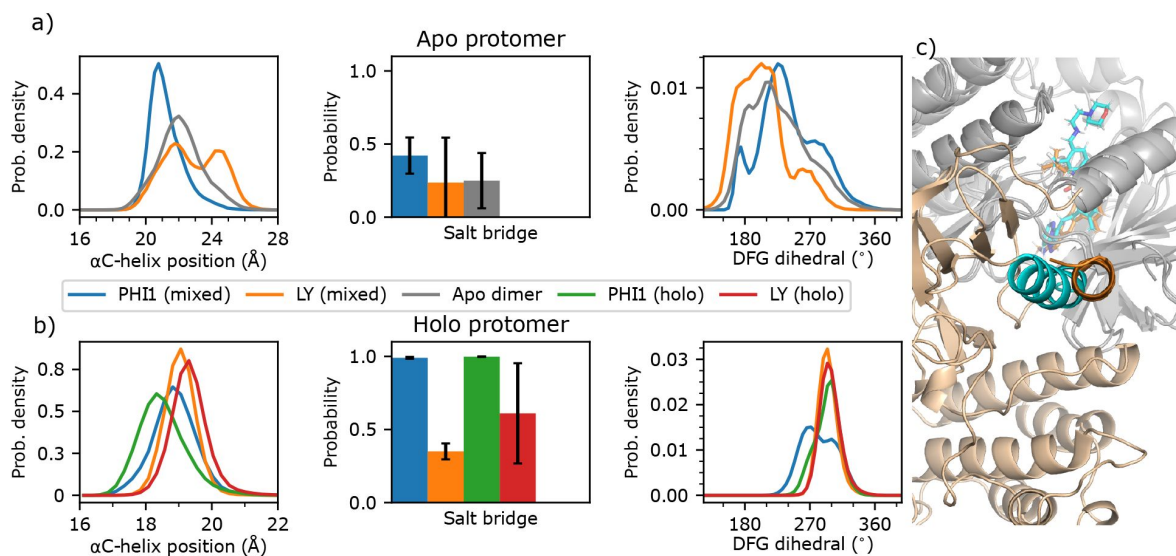


Figure 5.

Conformation of the α C helix and DFG motif is dependent on the presence or absence of PHI1 in the second protomer.

a) The α C helix position, probability of the Glu501–Lys483 salt bridge, and DFG pseudo dihedral of the apo protomer in the one PHI1- (blue) or one LY-bound (orange) mixed dimer simulations. As a reference, the apo dimer data is shown in grey. **b)** The same quantities as in a) but for the holo protomer in the one PHI1- (blue) or LY-bound (orange) mixed dimer simulations. As a reference, the two PHI1- and LY-bound holo dimer data are shown in green and red, respectively. The standard deviation of the probability across replicas is shown in error bars. **c)** Snapshot from both mixed dimers, after aligning the PHI1- (cyan) and LY-bound (orange) holo protomers (gray). The α C-helix of the apo protomer is highlighted in cyan for PHI1-bound and orange for LY-bound mixed dimer. For simplicity, only the apo protomer from the PHI1-bound mixed dimer is shown.

dimeric DFG is capable of occasionally sampling the DFG-in state due to the increased flexibility (Figure 3c). While this does suggest dimerization loosens the DFG motif, our simulations do not appropriately model the DFG-out/-in transition as the DFG-in state is only occasionally sampled (Supplemental Figure 5).

PHI1 and LY binding induces the α C-in state to varying degrees and shifts DFG out

Having understood how dimerization modulates the conformational dynamics of the α C helix and DFG motif, we proceeded to explore conformational changes induced by the dimer-compatible inhibitors PHI1 and LY. Interestingly and as expected, both inhibitors further restrain the motion of the α C helix, with its position sampling a narrower range of 4 Å, as compared to 7 Å in the apo dimer (Figure 3d, Supplemental Figure 6). Importantly, the α C position is shifted inward by at least 2.7 Å in the holo relative to the apo dimer, and PHI1 induces a larger shift, to 18.3 Å as compared to 19.3 Å in the presence of LY (Figure 3d, Supplemental Figure 6). The inward shift of the α C helix by the two inhibitors is also reflected in the stabilization of the Glu501–Lys483 salt bridge, which is promoted in the presence of LY (60% vs. 25% in the apo dimer) and is completely locked in the presence of PHI1 (Figure 3e, Supplementary Table 3). Although the DFG motif is also significantly restrained through inhibitor binding, the DFG pseudo dihedral in the holo dimer is shifted outward by 80° in complex with either PHI1 or LY (210° in the apo dimer vs. 290° in the holo dimer, Figure 3f, Supplemental Figure 6).

H-bond formation with Glu501 is critical for dimer selectivity by shifting α C helix inward

The monomer-selective inhibitors do not contact the center of the α C helix and their co-crystal structures only adopt α C-out state (Figure 2b). To test our hypothesis that the h-bond formation with Glu501 is critical for restricting the α C helix to the α C-in states, we examined the density plots of the α C position vs. the distance between the amide nitrogen of PHI1 or LY and the carboxylate of Glu501 in the holo dimer simulations (Figure 3g,h, Supplemental Figure 7). In the PHI1-bound dimer simulations, the PHI1–Glu501 h-bond is stable with only occasional breakages, as seen from the density maximum centered at the N4–Glu501 distance of 2.9 Å and α C position ~18 Å (Figure 3g, Supplemental Figure 7). In the LY-bound dimer simulations, however, the LY–Glu501 h-bond is weaker and less stable than the counterpart of the PHI1-bound dimer, as seen from the local density maximum centered at ~3.4 and the global maximum near ~4.5 Å (Figure 3g,h, Supplemental Figure 7). The stronger h-bond between PHI and Glu501 may be attributed to the additional hydrophobic interaction PHI1 forms with Asn500, which is absent for LY (Figure 2b). It is also noteworthy that when the PHI1–Glu501 interaction switches from h-bonding to van der Waals interaction, the α C position is slightly shifted outward to ~19 Å, which is similar to the position adopted in the LY-bound dimer simulations. This suggests that the stronger h-bond between PHI1 Glu501 may contribute to the inward α C position as compared to the LY-bound dimer.

To further dissect the mechanism of dimer selectivity, we examined the h-bond interaction between PHI1 or LY and Glu501 in inhibited monomer BRAF^{V600E} simulations. Strikingly, the PHI1–Glu501 interaction can become completely disrupted, with the distance moving beyond 6 Å to as high as 12 Å; correlated with the disruption of the PHI1–Glu501 interaction, the α C position is shifted out to the range of 21 Å–24 Å, similar to that sampled by the apo dimer (Figure 3i, Supplemental Figures 7,8). In stark contrast, the LY–Glu501 interaction remains stable as in the holo dimer simulations (Figure 3j, Supplemental Figures 7,8). These data are consistent with the previous simulations of the LY-and regorafenib (REG)-bound monomeric and dimeric BRAF^{V600E}

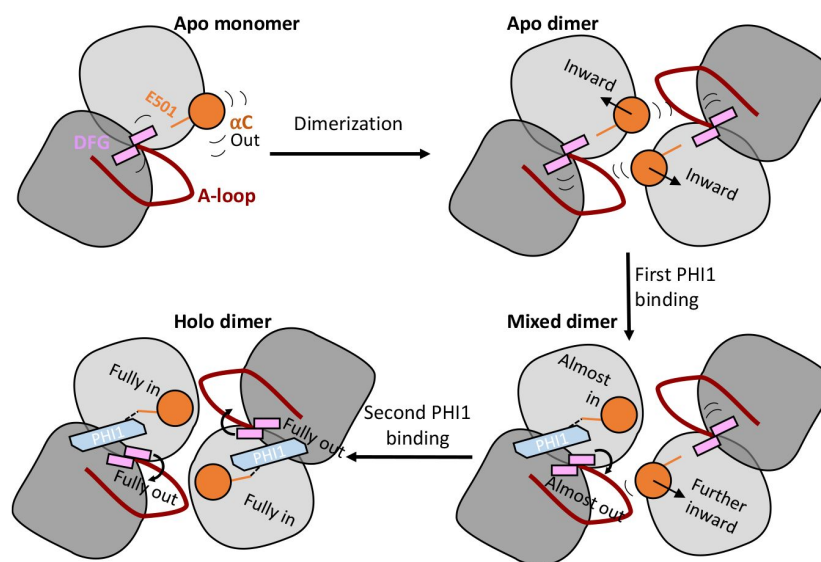


Figure 6.

A working model that explains dimer selectivity and binding cooperativity of BRAF^{V600E} inhibitors.

Top left. In the monomeric BRAF^{V600E}, the α C-helix (orange) is very flexible and exclusively samples the out states. **Top right.** Upon dimerization, the α C-helix is restrained and shifts inward, while the DFG-motif maintains its conformation but gains significant flexibility. **Bottom right.** When the first PH11 molecule binds, its amide linker donates a h-bond to the carboxylate of Glu501 (orange stick) in the first protomer, which locks the α C helix to the α C-in state; it also shifts and restricts the DFG-motif into the DFG-out state through the interaction with the DFG-Asp backbone. The α C-helix in the second unbound protomer is also affected, shifting in the direction of the inhibitor-bound state towards α C-in. Thus, the allosteric pre-organization primes the second protomer for accepting a second PH11 molecule. **Bottom left.** When the second PH11 molecule binds, the α C helix and DFG-motif in both protomers are shifted and fully locked into the α C-in and DFG-out states.

based on different force fields, which showed that the root-mean-square deviation (RMSD) of the dimer-selective REG is increased in the monomer compared to dimer simulations, whereas the RMSD of the equipotent LY remains the same (Adamopoulos et al., 2021 [↗](#)).

The correlation between the α C position and the LY–Glu501 interaction confirms our hypothesis that the h-bond interaction between the inhibitor and Glu501 is a key for restraining the α C helix and shifting it to the α C-in states. Since dimerization already restricts the motion of the α C helix and shifts it inward in the apo dimer, inhibitors capable of interacting with Glu501 can bind to the dimer via a conformational selection mechanism in addition to induced fit. On the other hand, conformational selection cannot be exploited for these inhibitors to bind the monomer, as the α C position in the apo monomer is outward. Compared to the equipotent inhibitors, the dimer-selective inhibitors such as PHI1 form much stronger h-bond with Glu501, which shifts the α C helix further inward. The latter may lead to a larger entropic penalty for the monomer binding as compared to the equipotent inhibitors.

PHI1 or LY binding has similar stabilizing effect on the dimer interface of BRAF^{V600E}

The aforementioned data demonstrates the importance of considering entropic penalty in monomer binding as a contributor to dimer selectivity. To rule out the possibility that the different degree of dimer (de)stabilization may also be a contributing factor for dimer selectivity, we turned to the difference contact network analysis (Yao et al., 2018 [↗](#)). In this analysis, the BRAF^{V600E} dimer was first partitioned into four different communities based on the the residue-residue contacts, which resulted in each community largely corresponding to the N-lobe (blue or grey) and C-lobe (red or orange) of either protomer (**Figure 4** [↗](#) left). Then, a community-community difference contact network between the apo and holo dimer simulation sets was calculated and mapped onto a graph, where the vertices represent the communities and blue and red edges represent the increased and decreased contact probabilities due to inhibitor binding (**Figure 4** [↗](#) right). Since we are interested in testing the dimer stability in the presence of PHI1 or LY, the interprotomer contact probabilities (between N-lobe:A and N-lobe:B or C-lobe:B; between C-lobe:A and C-lobe:B or N-lobe:A) were calculated and summed up. Interestingly, for both PHI1 and LY, the total interprotomer contact probability is increased (by 0.3) in the holo relative to the apo simulations. This net increase is mainly due to the N-lobe:A to C-lobe:B interactions which compensates for the decrease in the C-lobe:A to C-lobe:B contacts. This analysis demonstrates that both the dimer-selective and equipotent inhibitors have the same slightly stabilizing effect on the BRAF^{V600E} dimer interface; this rules out the possibility that the dimer selectivity is due to the different degree of dimer stabilization between the dimer-selective and equipotent inhibitors.

Positive cooperativity of PHI1 is due to the allosteric modulation of the α C and the DFG conformation in the opposite protomer

As previously mentioned, PHI1 was found to exert a more potent inhibition of the second protomers of the BRAF^{V600E} dimer whereas LY demonstrated similar potency in the inhibition of the two protomers (Cotto-Rios et al., 2020 [↗](#)). To shed light on this cooperativity mechanism, we examined the simulations of the mixed BRAF^{V600E} dimers in which only one protomer is in complex with PHI1 or LY. We first compared the α C helix position of the apo protomer in the mixed dimers (**Figure 5a** [↗](#), Supplemental Figure 9). Surprisingly, the α C helix of the apo protomer in the PHI1-bound mixed dimer is restrained and shifted inward by 1 Å relative to the apo dimer; in contrast, the position of the corresponding α C helix in the LY-bound mixed dimer remains the same but becomes slightly more flexible (blue and grey, **Figure 5a** [↗](#) left, Supplemental Figure 9). Consistent with the inward shift of the α C helix, the Glu501–Lys483 salt bridge has a lower average probability and a larger fluctuation in the apo dimer and the apo protomer of the LY-mixed dimer, as compared to the apo protomer of the PHI1-mixed dimer (blue and grey, **Figure 5a** [↗](#) middle).

These data suggest that PHI1 binding in one protomer allosterically modulates the α C helix in the second apo protomer such that it moves inward and becoming more favorable for binding the second PHI1.

While PHI1 or LY binding clearly perturbs the α C helix of the opposite apo protomer, the effect on the DFG conformation is less clear when comparing the DFG dihedral distribution of the apo protomer in the PHI1 or LY-mixed dimer with that of the apo dimer (blue, orange, and grey, **Figure 5a** right). All three distributions are broad, covering a range of 160–330°. It appears that, relative to the apo dimer, the DFG of the apo protomer in the PHI1-mixed dimer is slightly shifted to the right, whereas that of the LY-mixed dimer is slightly shifted to the left; however, these differences are very subtle and warrant further investigation in future studies.

Next, we compared the α C helix position in the holo protomer of the mixed dimers (**Figure 5b** left, Supplemental Figure 9). Remarkably, the α C helix in the PHI1-bound protomer of the mixed dimer (blue) is shifted outward by ~ 1 Å relative to the holo dimer bound to two PHI1 (green, **Figure 5b** left), demonstrating that the second PHI1 binding allosterically shifts the α C helix further inward. Further analysis shows that in the holo dimer the α C helix of one protomer is on average 0.5 Å closer compared to the neighboring protomer (Supplementary Table 2). Nonetheless, the Lys483–Glu501 salt bridge is stable in both the mixed and holo dimers; this is because the α C helix predominantly samples the α C-in state in both cases (blue and green in **Figure 5b** middle). In contrast to PHI1, the α C position in the LY-bound protomer of the mixed dimer is similar to that in the LY-bound holo dimer (orange and red in **Figure 5b** left), although the probability of the Lys483–Glu501 salt-bridge in the LY-bound protomer in the mixed dimer is slightly lower than in the holo dimer.

Consistent with the change in the DFG conformation between the holo (two inhibitor) and apo dimers (**Figure 3c,f**), DFG is rigidified upon binding of the first inhibitor, as evident from the narrower DFG dihedral distribution of the PHI1 or LY-bound protomer in the mixed protomer (**Figure 5b** right) compared to the apo protomer in the mixed dimer (**Figure 5a** right). Importantly, the DFG dihedral is right shifted in the occupied vs. apo protomer, demonstrating that the inhibitor pushes the DFG outward.

Consistent with the effect of the second PHI1 on the α C position of the first PHI1-bound protomer, binding of the second PHI1 shifts the peak of the DFG distribution for both protomers further outward, as shown by the 30° larger DFG pseudo dihedral in the holo dimer relative to the mixed dimer (green and blue in **Figure 5b** right; Supplementary Figures 6,9). In contrast, there is no significant difference in the DFG pseudo dihedral between the LY-mixed and holo dimers. These data suggest that while the binding of the first PHI1 pushes the DFG outward, binding of the second PHI1 has an allosteric effect, shifting the DFG of the opposite protomer further outward.

Concluding Discussion

We explored the mechanism of dimer selectivity and cooperativity of BRAF^{V600E} inhibitors using MD simulations of the dimeric and monomeric BRAF^{V600E}, in the absence and presence of one or two dimer-selective (PHI1) or equipotent (LY) inhibitor(s). The simulations uncovered the atomic details of the remarkable allostery in BRAF^{V600E} dimerization and ligand binding (**Figure 6**), which offer explanation for why some BRAF inhibitors are monomer selective while others are dimer compatible, i.e., selective or equipotent. Specifically, our data showed that dimerization of BRAF^{V600E} leads to the restriction and an inward shift of the α C helix position relative to the monomer (**Figure 6** top panel), which explains why inhibitors that can stabilize the α C-in states are dimer compatible whereas those that cannot are monomer selective. The fact that both

dimerization and inhibitor binding induces α C to move inward contributes to the phenomenon of drug-induced RAF dimerization (Hatzivassiliou et al., 2010 [↗](#); Lavoie et al., 2013 [↗](#); Karoulia et al., 2016 [↗](#)).

The co-crystal structure analysis and MD simulations identified a h-bond donor (e.g., an amide linker in the dimer-selective PHI1 or the equipotent LY) as a key for dimer compatibility; the h-bond with the carboxylate of Glu501 stabilizes the α C helix in the α C-in states. Two factors make Glu501 a special and critical anchoring point for inducing the α C-in states. First, it is located at the center of the α C helix, which makes it easier (as opposed to the end of the helix) to induce a helix movement. Second, the h-bonding between the inhibitor and Glu501 is synergistic with the Lys483–Glu501 salt-bridge formation. In contrary, the lack of a h-bond with Glu501, e.g., in VEM, Debrafenib, or PLX7904, results in the monomer selectivity. Note, the DFG-in inhibitors can also donate a h-bond to α C-Glu501 and bind the BRAF^{V600E} dimer. An example is SB5909885, which donates a h-bond from the oxime group to α C-Glu501 and also forms a salt bridge with Lys483 (PDB ID: 2FB8) (King et al., 2006 [↗](#)).

The difference between the dimer-selective and equipotent inhibitors is more subtle. The MD simulations revealed that PHI1 forms a more stable h-bond with Glu501 in the BRAF^{V600E} dimer as compared to LY, which is consistent with the ~ 1 Å inward shift of the α C helix and more stable Lys483–Glu501 salt bridge. The latter differences are much smaller in the co-crystal structures; the α C positions and Lys483–Glu501 distances are only respectively 0.1 and 0.2 Å smaller in the PHI1- vs. LY-bound co-crystal structure. Since the monomeric BRAF^{V600E} has a flexible α C helix that predominantly samples the α C-out states, forming a tighter h-bond would incur a higher entropic penalty for monomer binding. This may explain why the PHI1–Glu501 interaction as well as the α C position are unstable in the monomer simulations but stable in the dimer simulations, in contrast to the LY-bound simulations. Therefore, the stability of the h-bonding with Glu501 may be a key for dimer selectivity.

Without the MD simulations, how would one determine if the h-bond between the inhibitor and Glu501 is stable? We found that the deviation between the α C position and/or K–E distance of the two protomers in the co-crystal structure offers some indication (Supplemental Table 1). With the exception of LY and Ponatinib, the α C position and/or K–E distance between the two protomers in the co-crystal structures of AZ628, TAK632, BGB283, SB5909885 deviate by 0.3 Å or higher (Supplemental **Table 1** [↗](#)). In contrast, the α C position and the K–E distance are (nearly) identical between the two protomers in the co-crystal structures of the dimer-selective inhibitors LXH254, RAF709, Sorafenib, and Belvarafenib (Supplemental Table 1). The identical α C position and K–E distance in the two protomers suggest that the α C helix is restrained by the inhibitor, i.e. it forms a stable h-bond with Glu501.

To additionally test this crystal structure-based hypothesis, we examined the co-crystal structures of GDC0879 and Tovorafenib, which were not analyzed in Ref (Adamopoulos et al., 2021 [↗](#)). In the co-crystal structure of GDC0879 (PDB ID: 4MNF), the α C position deviates by 0.3 Å and the K–E distance deviates by 0.1 Å between the two protomers. In the co-crystal structure of Tovorafenib (PDB ID: 6V34), the α C position deviates by 0.2 Å and the K–E distance deviates by 0.4 Å between the two protomers. These deviations suggest that the α C is not adequately restrained by the inhibitors and therefore we predicted GDC0879 and Tovorafenib to be equipotent. Note, GDC0879 is a DFG-in inhibitor, which is an additional indication for a equipotent inhibitor. Indeed, both GDC0879 and Tovorafenib were found as equipotent in experimental studies (Karoulia et al., 2016 [↗](#); Tkacik et al., 2023 [↗](#)). These analyses led us to propose the following empirical assessment of a RAF inhibitor based on its co-crystal structure with BRAF^{V600E}: 1) lack of a h-bond with Glu501 indicates monomer selectivity; 2) presence of a h-bond with Glu501 but inconsistent α C position and/or K–E distance between the two protomers indicates equipotency; 3) presence of a h-bond with Glu501 and identical α C position and K–E distance between the two protomers indicates that

the inhibitor is likely (but not necessarily) dimer selective. Given that the resolution of a resolved structure is often $\sim 2\text{-}3\text{ \AA}$, this proposed assessment is not intended to replace more rigorous tests, i.e. utilizing MD simulations.

Finally, the MD analysis uncovered a mechanism for positive cooperativity. Our findings are summarized in **Figure 6**; upon dimerization (top row) the αC -helix goes from αC -out and highly flexible to slightly restrained and inward shifted. The first PHI1 binding in the BRAF^{V600E} dimer restricts the motion of the αC helix and DFG, shifting them slightly inward and outward, respectively (**Figure 6**, bottom right panel). Intriguingly, the first PHI1 binding primes the apo protomer by making the αC more favorable for binding, i.e., shifting the αC inward (**Figure 6**, bottom right panel). Importantly, upon binding the second PHI1, the αC helix is shifted further inward and the DFG is shifted further outward in both protomers. These data suggest that the positive cooperativity of PHI1 is due to its ability to allosterically modulate the αC and the DFG conformation in the second protomer. Taken together, our findings provide a mechanistic understanding for the remarkable allostery and conformational interplay between kinase dimerization and inhibitor binding. As we prepare the manuscript for submission, a biophysical experiment was published, which suggested that the first inhibitor binding dominates the allosteric coupling between type II inhibitor binding and BRAF dimerization (Rasmussen et al., 2023), consistent with our simulation data. The work presented here has implications for understanding the molecular mechanism of kinase signaling and contributes to the rational design of protomer-selective inhibitors.

Methods and Protocols

Intracellular homogeneous TR-FRET assay

SKMEL239 and SKMEL239-C4 cells were plated at 50000cells/well in white TC-treated 96-well plates in 100ul complete growth media (DMEM). Cells were incubated with the various RAF inhibitors for 1 hour at 37°C, 5% CO₂. ERK phosphorylation was measured using the THUNDER™ Extreme Phospho-ERK1/2 (T202/Y204) TR-FRET Cell Signaling Assay Kit (Bioauxillium) according to directions for the Standard 2-Plate Assay Protocol for Adherent Cells. Cells were lysed for 30 minutes at RT under shaking. Lysates were transferred to a white 384-well plate, sealed and incubated with the detection mix antibody at RT for 4 hours. TR-FRET signal was measured at 615 nm and 665 nm excitation using a TECAN SPARK plate reader.

System preparation for simulations

Simulations were prepared using a crystal structure of BRAF^{V600E} in complex with either PHI1 (PDB ID: 6P7G) (Cotto-Rios et al., 2020) or LY (PDB ID: 5C9C) (Peng et al., 2015). The initial structure of the apo simulations was taken from 6P7G. The a-loop is not resolved in either protomer in 5C9C, but is resolved for protomer B in 6P7G. Thus, the missing a-loop in the protomer B of 6P7G and in both protomers in 5C9C were built by rotating and translating the resolved a-loop from the first protomer using the alignment tool in PyMOL (Schrödinger, LLC, 2015). Chain B (which has the resolved a-loop) was first aligned to chain A using all residues except for the a-loop and the two end residues that connect it to the rest of the protein. Following the alignment, chain B except for the a-loop and its two end residues were deleted. The N-terminus was acetylated and the C-terminus was amidated. Hydrogen atoms were added using the HBUILD facility in the CHARMM package (version c37a2) (Brooks et al., 2009).

All-atom continuous constant pH molecular dynamics (CpHMD) simulations

The recently developed all-atom particle mesh Ewald CpHMD (PME-CpHMD) (Harris et al., 2022 [↗](#)) with the asynchronous pH replica exchange sampling protocol (Wallace and Shen, 2011 [↗](#); Henderson et al., 2020 [↗](#)) was used to determine the protonation and tautomer states of histidines. To prepare for the CpHMD simulations, the histidine residues were first set to HIP with the dummy hydrogens on the N δ and N ϵ atoms. The system was solvated in a rectangular water box with at least 10 Å distance between the protein and the boundary (~23,000 water molecules). The protein was represented by the AMBER ff14SB force field (Maier et al., 2015 [↗](#)) and water by the TIP3P model (Jorgensen et al., 1983 [↗](#)). The dimer structure was briefly minimized for 500 steps (first 200 were using steepest decent, following 300 used conjugate gradient) with a harmonic force constant of 100 kcal/mol/Å² applied on all heavy atoms of the protein. This was followed by 100 ps of heating to 300 K using the PME-CpHMD simulations at pH 7.0 with the restraints still applied. Once heated the restraints were gradually removed in six stages: in the first two stages the protein heavy atoms were restrained with a force constant of 100 and 10 kcal/mol/Å²; in the next four stages only the backbone heavy atoms were restrained with a force constant of 10, 1.0, 0.1, and 0.0 kcal/mol/Å. Each stage was simulated for 250 ps, for a total of 1.5 ns. A cutoff of 12 Å was used for the non-bonded interactions.

The equilibrated structure was then used to initiate the pH replica exchange PME-CpHMD simulations. The asynchronous pH replica exchange sampling protocol (Wallace and Shen, 2011 [↗](#); Henderson et al., 2020 [↗](#)) was used to accelerate convergence of the coupled protonation and conformational states (Wallace and Shen, 2011 [↗](#)). Five replicas were created at different pH conditions, from pH 6.5 to 8.5. Each replica was first equilibrated to its pH by repeating the final four stages of equilibration mentioned above. The pH replica exchange CpHMD was then conducted for 10 ns with attempted swaps of neighboring pH conditions occurring every 2 ps. All other settings are identical to Ref. *Harris et al. (2022)*. For the calculation of protonation and tautomer state probabilities, the λ and x values above 0.8 or below 0.2 were used (default setting in the CpHMD analysis package (Henderson et al., 2022 [↗](#))). At pH 7.5 His477 was protonated at both N ϵ and N δ while His510 was protonated at N ϵ only. These protonation/tautomeric states were used for all convention (fixed-protonation-state) simulations below.

Conventional fixed-protonation-state MD simulations

Eight BRAF^{V600E} systems were simulated, consisting of monomeric and dimeric BRAF^{V600E} either in the presence or absence of PHI1 or LY (see **Table 1** [↗](#)). Monomer systems were prepared by eliminating one protomer from the prepared dimer structure. In the apo monomer and dimer systems, ligand(s) was removed. In the mixed or holo systems, one or both inhibitors from the co-crystal structure was kept. The protein was then placed in a rectangular water box with a minimum distance of 10 Å between the protein and edges of the water box using the LEaP program (Case et al., 2020 [↗](#)). Based on the protonation states determined using CpHMD, sodium and chloride ions were added to neutralize the system and reach a physiological ionic strength of 0.15 M.

The conventional (fixed-protonation-state) MD simulations were carried out using the AMBER20 MD package (Case et al., 2020 [↗](#)). The proteins was represented by the ff14SB force field (Maier et al., 2015 [↗](#)) while inhibitors were parameterized by the general AMBER force field (GAFF) method (Wang et al., 2004 [↗](#)). The TIP3P model (Jorgensen et al., 1983 [↗](#)) was used to represent water. The Leapfrog integrator was used to propagate the coordinates. The SHAKE algorithm was applied to bonds involving hydrogen to allow for a 2-fs time step. Additionally, the hydrogen mass re-partitioning (Hopkins et al., 2015 [↗](#)) was used to redistribute the mass between hydrogens and their bonded heavy atoms to allow for a 4-fs time step. A nonbonded cutoff of 8 Å was used as in the ff14SB validation study (Maier et al., 2015 [↗](#)) while the electrostatic potentials were computed

using the particle-mesh Ewald method (Darden et al., 1993 [↗](#)) with a real-space cut-off of 12 Å and a sixth-order interpolation with approximately 1 Å grid spacing. Each system underwent minimization using 1000 steps of steepest descent followed by 19000 steps of conjugate gradient while the heavy atoms were harmonically restrained using a force constant of 100 kcal/mol/Å². Following minimization, the system was heated to 300 K over 1 ns under an NVT ensemble using a Langevin thermostat (Feller et al., 1995 [↗](#)) with collision frequency of 1 ps⁻¹ for temperature control. The systems then underwent a 6-stage equilibration in which the backbone restraints were gradually reduced to 10, 5, 2, 1, 0.1 and 0 kcal/mol/Å² over the course of 100 ns under a NPT ensemble. A Monte-Carlo barostat (Case et al., 2020 [↗](#)) was used to control pressure at 1 bar using a relaxation time of 1.0 ps. Each system were run in three replicates each starting from different random velocity seeds and each run lasted 5 μs.

Simulation data analysis

CPPTraj (Roe and Cheatham, 2013 [↗](#)) was used to analyze the protomer conformation (α C-helix position, DFG pseudo dihedral, etc.) and visualizations were produced using PyMOL (Schrödinger, LLC, 2015 [↗](#)). The contact network analysis was conducted using the open source code developed by Yao and Hamelberg (<https://github.com/The-Hamelberg-Group/dcna> [↗](#)) (Yao et al., 2018 [↗](#)). Unless otherwise noted, the last three μs trajectory frames were used for analysis. All probability distributions were created by combining the last three μs of each replica for each system, with each distribution consisting of 50 bins. Unless specified, distributions contain quantities from both protomers in dimeric simulations.

Data Availability

The MD simulation input files and analysis scripts are freely downloadable from <https://github.com/JanaShenLab/RAF/> [↗](#). The raw MD trajectories are available upon request.

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Additional files

Supporting Information [↗](#)

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Reviewer #1 (Public Review):

Summary:

This manuscript from Clayton and co-authors aims to clarify the molecular mechanism of BRAF dimer selectivity. Indeed, first generation BRAF inhibitors, targeting monomeric BRAFV600E, are ineffective in treating resistant dimeric BRAF isoforms. Here, the authors employed molecular dynamics simulations to study the conformational dynamics of monomeric and dimeric BRAF, in the presence and absence of inhibitors. Multi-microseconds MD simulations showed an inward shift of the α C helix in the BRAFV600E mutant dimer. This helped identify a hydrogen bond between the inhibitors and the BRAF residue Glu501 as critical for dimer compatibility. The stability of the aforementioned interaction seems to be important to distinguish between dimer-selective and equipotent inhibitors.

Strengths:

The study is overall valuable and robust. The authors used the recently developed particle mesh Ewald constant pH molecular dynamics, a state-of-the-art method, to investigate the correct histidines protonation considering the dynamics of the protein. Then, multi-microsecond simulations showed differences in the flexibility of the α C helix and DFG motif. The dimerization restricts the α C position in the inward conformation, in agreement with the result that dimer-compatible inhibitors are able to stabilize the α C-in state. Noteworthy, the MD simulations were used to study the interactions between the inhibitors and the protein, suggesting a critical role for a hydrogen bond with Glu501. Finally, simulations of a mixed state of BRAF (one protomer bound to the inhibitor and the other apo) indicate that the ability to stabilize the inward α C state of the apo protomer could be at the basis of the positive cooperativity of PHI1.

<https://doi.org/10.7554/eLife.95334.3.sa2>

Reviewer #2 (Public Review):

Summary:

The authors employ molecular dynamics simulations to understand the selectivity of FDA approved inhibitors within dimeric and monomeric BRAF species. Through these comprehensive simulations, they shed light on the selectivity of BRAF inhibitors by delineating the main structural changes occurring during dimerization and inhibitor action. Notably, they identify the two pivotal elements in this process: the movement and conformational changes involving the α -C helix and the formation of a hydrogen bond involving the Glu-501 residue. These findings find support in the analyses of various structures crystallized from dimers and co-crystallized monomers in the presence of inhibitors. The elucidation of this mechanism holds significant potential for advancing our understanding of kinase signalling and the development of future BRAF inhibitor drugs.

Strengths:

The authors employ a diverse array of computational techniques to characterize the binding sites and interactions between inhibitors and the active site of BRAF in both dimeric and monomeric forms. They combine traditional and advanced molecular dynamics simulation techniques such as CpHMD (All-atom continuous constant pH molecular dynamics) to provide mechanistic explanations. Additionally, the paper introduces methods for identifying and

characterizing the formation of the hydrogen bond involving the Glu501 residue without the need for extensive molecular dynamics simulations. This approach facilitates the rapid identification of future BRAF inhibitor candidates.

<https://doi.org/10.7554/eLife.95334.3.sa1>

Author response:

The following is the authors' response to the previous reviews.

Reviewer #1 (Public review):

Comment 1: This manuscript from Clayton and co-authors, entitled "Mechanism of dimer selectivity and binding cooperativity of BRAF inhibitors", aims to clarify the molecular mechanism of BRAF dimer selectivity. Indeed, first-generation BRAF inhibitors, targeting monomeric BRAFV600E, are ineffective in treating resistant dimeric BRAF isoforms. Here, the authors employed molecular dynamics simulations to study the conformational dynamics of monomeric and dimeric BRAF, in the presence and absence of inhibitors. Multi-microsecond MD simulations showed an inward shift of the α C helix in the BRAFV600E mutant dimer. This helped in identifying a hydrogen bond between the inhibitors and the BRAF residue Glu501 as critical for dimer compatibility. The stability of the aforementioned interaction seems to be important to distinguish between dimer-selective and equipotent inhibitors.

The study is overall valuable and robust. The authors used the recently developed particle mesh Ewald constant pH molecular dynamics, a state-of-the-art method, to investigate the correct histidine protonation considering the dynamics of the protein. Then, multi-microsecond simulations showed differences in the flexibility of the α C helix and DFG motif. The dimerization restricts the α C position in the inward conformation, in agreement with the result that dimer-compatible inhibitors can stabilize the α C-in state. Noteworthy, the MD simulations were used to study the interactions between the inhibitors and the protein, suggesting a critical role for a hydrogen bond with Glu501. Finally, simulations of a mixed state of BRAF (one protomer bound to the inhibitor and the other apo) indicate that the ability to stabilize the inward α C state of the apo protomer could be at the basis of the positive cooperativity of PHI1.

We thank the reviewer for the positive evaluation of our work.

Comment 2a: Regarding the analyses of the mixed state simulations, the DFG dihedral probability densities for the apo protomer (Fig. 5a right) are highly overlapping. It is not convincing that a slight shift can support the conclusion that the binding in one protomer is enough to shift the DFG motif outward allosterically. Moreover, the DFG dihedral time-series for the apo protomer (Supplementary Figure 9) clearly shows that the measured quantities are affected by significant fluctuations and poor consistency between the three replicates. The apo protomer of the mixed state simulations could be affected by the same problem that the authors pointed out in the case of the apo dimer simulations, where the amount of sampling is insufficient to model the DFG-out/-in transition properly.

While the reviewer is correct there are large fluctuations in the DFG pseudo dihedral over the course of the apo simulations, these fluctuations occur primarily in the first 2 μ s of the simulations, which were removed from our analysis. The reviewer is also correct that these simulations do not sufficiently model the DFG-out/-in transition; however, a full transition is not necessary for our analysis, as we are only interested in the shift of the DFG pseudo

dihedral. As to the reviewer's comment on the overlapping DFG distributions, we agree that the difference is very subtle. We revised the text.

On page 9, second paragraph from the bottom:

“While PHI1 or LY binding clearly perturbs the α C helix of the opposite apo protomer, the effect on the DFG conformation is less clear when comparing the DFG dihedral distribution of the the apo protomer in the PHI1 or LY-mixed dimer with that of the apo dimer (blue, orange, and grey, Figure 5a right). All three distributions are broad, covering a range of 160-330°. It appears that, relative to the apo dimer, the DFG of the apo protomer in the PHI1-mixed dimer is slightly shifted to the right, whereas that of the LY-mixed dimer is slightly shifted to the left; however, these differences are very subtle and warrant further investigation in future studies.”

Comment 2b: There is similar concern with the Lys483-Glu501 salt bridge measured for the apo protomers of the mixed simulations. As it can be observed from the probabilities bar plot (Fig. 5a middle), the standard deviation is too high to support a significant role for this interaction in the allosteric modulation of the apo protomer.

As for the salt bridge, the fluctuation in the apo dimer and LY-mixed dimer is indeed large, and together with the lower average probability suggests that the salt bridge is weaker, which is consistent with the α C helix moving outward. To clarify this, we revised the text.

On page 9, second paragraph from the bottom:

“Consistent with the inward shift of the α C helix, the Glu501–Lys483 salt bridge has a lower average probability and a larger fluctuation in the apo dimer and the apo protomer of the LY-mixed dimer, as compared to the apo protomer of the PHI1-mixed dimer.”

Reviewer #2 (Public review):

Comment 1: The authors employ molecular dynamics simulations to understand the selectivity of FDA approved inhibitors within dimeric and monomeric BRAF species. Through these comprehensive simulations, they shed light on the selectivity of BRAF inhibitors by delineating the main structural changes occurring during dimerization and inhibitor action. Notably, they identify the two pivotal elements in this process: the movement and conformational changes involving the alpha-C helix and the formation of a hydrogen bond involving the Glu-501 residue. These findings find support in the analyses of various structures crystallized from dimers and co-crystallized monomers in the presence of inhibitors. The elucidation of this mechanism holds significant potential for advancing our understanding of kinase signalling and the development of future BRAF inhibitor drugs.

The authors employ a diverse array of computational techniques to characterize the binding sites and interactions between inhibitors and the active site of BRAF in both dimeric and monomeric forms. They combine traditional and advanced molecular dynamics simulation techniques such as CpHMD (all-atom continuous constant pH molecular dynamics) to provide mechanistic explanations. Additionally, the paper introduces methods for identifying and characterizing the formation of the hydrogen bond involving the Glu501 residue without the need for extensive molecular dynamics simulations. This approach facilitates the rapid identification of future BRAF inhibitor candidates.

We thank the reviewer for the positive evaluation of our work.

Comment 2: Despite the use of molecular dynamics yields crucial structural insights and outlines a mechanism to elucidate dimer selectivity and cooperativity in these systems, the authors could consider adoption of free energy methods to estimate the values of hydrogen bond energies and hydrophobic interactions, thereby enhancing the depth of their analysis.

As mentioned in our previous response, current free energy methods are capable of giving accurate estimates of the relative binding free energies of similar ligands; however, accurate calculations of the absolute free energies of hydrogen bond and hydrophobic interactions are not feasible yet. Thus, we decided not to pursue the calculations.

Reviewer #1 (Recommendations to author):

Comment 1: It would be useful to cite all supplementary figures in the main text (where relevant). In the present version, only Supplementary Figures 2,3, and 4 are cited in the main text.

This was an oversight; supplementary figures 5 through 9 are now cited in the text, to point to the time-series of the quantity discussed. We note that supplementary figures 10 and 11 show the time-series of the root mean squared deviation (RMSD) of each protomer in both all monomeric and dimeric simulations; these quantities are not discussed in the manuscript but are provided for further insight.

Comment 2: It is unclear whether the present data could support a direct involvement of the DFG movement in the allosteric mechanism proposed. The same argument applies to the Lys483Glu501 interaction in the apo protomer of the mixed state simulations. The current simulation data could only support a different stabilization of the α C-helix position. The authors should either remove/tone down the claim or extend the simulations to sample a “converged” distribution of the DFG dihedral and the Lys483-Glu501 salt bridge of the apo protomers.

We agree that the DFG change in the apo protomer of the PH1-mixed dimer is very subtle (see our response and revision to comment 2); however, the allosteric involvement of DFG is clearly demonstrated in Figure 5 (right panel in 5a and 5b). We compare three states: apo protomer in the mixed dimer, PH1-bound protomer in the mixed dimer, and holo dimer (i.e., with two PH1). Binding of the first PH1 restricts the DFG conformation to the larger DFG dihedrals (blue curves in the top and bottom right panels). This effect (DFG outward and more restricted) is even strong when the second PH1 binds, locking the DFG in both protomers to a narrow dihedral range 270–330 degree (green and blue curves in Figure 5b, right panel). These are allosteric effects, demonstrating that the second PH1 binding induces conformational change of the DFG in the first protomer. This is why in Figure 6, the DFG of the PH1-bound protomer in the mixed dimer is labeled as “almost out”, while the DFG in the holo dimer is labeled as “fully out”.

The effect of second PH1 on the DFG of the first protomer is consistent with that the α C helix position, in which case, the second PH1 induces an inward movement of the α C of the first protomer (illustrated as “fully in” in the schematic Figure 6). Through the α C movement, the salt-bridge strength is affected, as we discussed in our response and revision to Reviewer’s comment 2a. To clarify these points, we revised the discussion of Figure 5. We made the x axis range of the DFG dihedral distributions the same between the top and bottom panels in Figure 5. To remove the claim of priming effect on DFG, we revised Figure 6.

Page 10, Figure 5:

we made the x axis range of the DFG dihedral distributions on the top and bottom panels the same to facilitate comparison.

Page 11, second and third paragraphs:

“Consistent with the change in the DFG conformation between the holo (two inhibitor) and apo dimers (Figure 3c,3f), DFG is rigidified upon binding of the first inhibitor, as evident from the narrower DFG dihedral distribution of the PHI1 or LY-bound protomer in the mixed protomer (Figure 5b right) compared to the apo protomer in the mixed dimer (Figure 5a right). Importantly, the DFG dihedral is right shifted in the occupied vs. apo protomer, demonstrating that the inhibitor pushes the DFG outward.”

“Consistent with the effect of the second PHI1 on the α C position of the first PHI1-bound protomer, binding of the second PHI1 shifts the peak of the DFG distribution for both protomers further outward, as shown by the 30° larger DFG pseudo dihedral in the holo dimer relative to the mixed dimer (green and blue in Figure 5b right; Supplementary Figures 6,9). In contrast, there is no significant difference in the DFG pseudo dihedral between the LY-mixed and holo dimers. These data suggest that while the binding of the first PHI1 pushes the DFG outward, binding of the second PHI1 has an allosteric effect, shifting the DFG of the opposite protomer further outward.”

On page 12, the last paragraph of Conclusion, we remove the claim of the priming effect for DFG:

“The first PHI1 binding in the BRAF^{V600E} dimer restricts the motion of the α C helix and DFG, shifting them slightly inward and outward, respectively (Figure 6, bottom right panel). Intriguingly, the first PHI1 binding primes the apo protomer by making the α C more favorable for binding, i.e., shifting the α C inward (Figure 6, bottom right panel). Importantly, upon binding the second PHI1, the α C helix is shifted further inward and the DFG is shifted further outward in both protomers.”

On page 13, Figure 6:

we removed the label “slightly outward” for DFG.

Comment 3: An alternative approach could be using enhanced sampling methods to enhance the diffusion along these coordinates.

We thank the reviewer for bringing up this point. While that the allostery and cooperativity effects are apparent from our simulation data, we agree that enhanced sampling methods in principle could be used to further converge the conformational sampling; however, these approaches face significant challenges. First, BRAF dimer is weakly associated, with α C helix forming a part of the dimer interface. Enhanced sampling of α C helix would likely result in dimer dissociation. On the other hand, simply using RMSD as a reaction coordinate or progress variable would not necessarily enhance the motion of α C helix or DFG or activation loop, which are all coupled. Second, our extensive simulations of a monomer kinase with metadynamics demonstrated that the kinase conformation becomes distorted when a biasing potential is placed to enhance the motion of DFG. This is likely because the other parts of the protein do not have enough time to relax to accommodate the conformational change. To our knowledge, this aspect has not been discussed in the current metadynamics literature, which focuses on the free energy differences and (local) conformational changes along the reaction coordinate. To clarify these points, we added a discussion.

Page 6, end of the first paragraph:

“We note that enhanced sampling methods were not used due to several challenges. First, the BRAF dimer is weakly associated, with α C helix forming a part of the dimer interface (Figure 1a). Enhanced sampling (particularly of α C helix) would likely lead to dimer dissociation. Second, biased sampling methods such as metadynamics may lead to unrealistic conformational states due to the slow relaxation of some parts of the protein to accommodate the conformational change directed by the reaction coordinate. For example, our unpublished metadynamics simulations of a monomer kinase showed that enhancing the DFG conformational change resulted in distortion of the kinase structure.”

We thank the reviewers again for their valuable comments. We believe our revision has further elevated the quality of the manuscript.

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