Protomer Selectivity of RAF Inhibitors Within the RAS/RAF Signalosome

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Abstract

RAF dimer inhibitors offer therapeutic potential in RAF- and RAS-driven cancers. The utility of such drugs is predicated on their capacity to occupy both RAF protomers in the RAS-RAF signaling complex. Here we describe a method to conditionally quantify drug-target occupancy at selected RAF protomers within an active RAS-RAF signalosome in cells. RAF target engagement can be measured in the presence or absence of any mutant KRAS allele, enabling the high affinity state of RAF dimer inhibitors to be quantified in the cellular milieu. The intracellular selectivity of clinical-stage drugs for individual protomers within BRAF, CRAF, and ARAF heterodimers in complex with mutant KRAS-GTP revealed that ARAF protomer-engagement, but not engagement of BRAF or CRAF is commensurate with inhibition of MAPK signaling in various mutant RAS cell lines. Our results support a fundamental role for ARAF in mutant RAS signaling and highlight the avoidance of ARAF protomers for a cohort of RAF inhibitors undergoing clinical evaluation.

Introduction

The RAS/RAF/MEK/ERK signaling cascade is a master regulatory pathway controlling cell growth, proliferation, and survival\(^1\). Normal pathway agonism triggers the association of GTP-bound RAS (KRAS, HRAS, NRAS) and the primary pathway effectors, RAF kinases (BRAF, CRAF, ARAF), via their N-terminal regulatory domains\(^2\). RAS-RAF interactions stabilize the RAF kinase domain in an αC helix-IN conformation and facilitates dimerization. All combinations of RAF homodimerization and heterodimerization can occur, depending on the cellular context\(^1,3\). The active, membrane-tethered RAS/RAF signaling complex phosphorylates MEK, which, in turn, phosphorylates ERK – resulting in the activation of multiple transcription factors. Genetic lesions in this pathway are observed in greater than 30% of human cancers, primarily via activating mutations in KRAS, NRAS, and BRAF\(^1,4\). Drugging RAS, however, is notoriously challenging due to the scarcity of binding pockets on this small GTPase, coupled with its exceedingly strong affinity for guanosine nucleotides\(^4,5\). Therefore, efforts to target the catalytic activity of kinases in the pathway have been intensely pursued for several decades. Somatic mutations and fusions of all RAF paralogs are detected in tumors, but three classes of BRAF mutations account for most oncogenic RAF mutations\(^6,7\). BRAF Class I mutations (2.5% of all cancers) occur at the V600 codon and produce a constitutively active monomeric kinase. Class II mutations (0.7% of all cancers) are defined as non-V600 variants that activate BRAF signaling via RAS-independent dimers. And Class III mutations (0.7% of all cancers) have decreased intrinsic kinase activity compared to wild type BRAF, but increased RAS-dependent heterodimerization and activation of CRAF\(^1\).

Initial drug discovery efforts targeting BRAF Class I mutants have been successful in treating melanoma expressing BRAF V600E, but resistance inevitably occurs, often within 6 months of treatment\(^8,9\). Drug-resistance to the marketed inhibitors (vemurafenib, dabrafenib, encorafenib) is largely attributed to feedback amplification of RAF signaling through canonical pathway members or the activation of other oncogenic pathways (e.g., PI3K/AKT)\(^10–16\). The increase in RAF signaling is due to upregulation of
receptor tyrosine kinases (e.g., EGFR), activating RAS mutations, alternative dimer inducing BRAF mutations, or overexpression of mutant BRAF. Notably, there is also a feedback inhibition of RAS/RAF by ERK, and therefore blocking V600E-driven ERK signaling alleviates this repression of wildtype RAF. Additionally, in cells harboring wildtype BRAF, treatment with FDA-approved BRAF V600E inhibitors fails to inhibit, and instead induces RAS-GTP-dependent agonism of mitogenic ERK signaling. This so called “RAF inhibitor paradox” is a result of BRAF dimerization and negative allostery, which prevents the drug from binding to the second, activated RAF protomer. Consequently, the selectivity of these inhibitors for monomeric BRAF precludes their broader utility in RAS-mutant tumors and limits their therapeutic index in a wildtype RAF setting.

Concerted, multidisciplinary efforts have yielded two pharmacological strategies to circumvent first-generation RAF inhibitor (RAFi)-resistance via upregulation of the canonical wildtype pathway components and the paradoxical dimerization and transactivation of RAF kinases. These drug candidates stabilize DFG-IN or OUT and αChelix IN or OUT conformations of the kinase domain and are categorized accordingly (Fig. 1A). “Paradox breakers” are a class of Type 1.5, DFG-IN, αC-OUT BRAF inhibitors that bind mutant and wildtype BRAF monomers and block their dimerization by altering the dimer interface of the αC-helix. Conversely, “RAF dimer inhibitors” (also called “pan-RAF inhibitors” in literature) antagonize RAF signaling despite promoting and stabilizing RAF dimers, by occupying both protomers of the complex. RAF dimer inhibitors are predominantly Type II kinase inhibitors that engage BRAF and CRAF in a DFG-OUT, αC-helix-IN conformation. These inhibitors may offer superior therapeutic utility for MAPK-pathway inhibition in RAS-driven tumors, since drug engagement is expected to be enhanced by higher levels of RAS-GTP.

BRAF and CRAF are the most catalytically active of the three RAF paralogs and are generally the focus of next generation RAF inhibitor development programs. Engagement of ARAF is often unreported. However, ARAF kinase activity has been recently implicated in RAS-driven tumors. For example, in multiple tumor cell types harboring wildtype RAFs and mutant RAS, treatment with the RAF dimer inhibitor naporafenib (LXH254) results in relatively weak MAPK-pathway inhibition and antiproliferative effect. Despite potent inhibition of all three RAF paralogs with LXH254 in a cell-free context, analysis of cell lysates suggest that engagement of ARAF may be weaker than BRAF or CRAF in mutant a KRAS setting. Supporting a critical role for ARAF, genetic ablation of ARAF sensitized cells to LXH254 in several mutant KRAS-driven lineages (G12C, G13D). Moreover, quantitative proteomic studies showed that RAS-bound ARAF:CRAF heterodimers are significantly upregulated relative to BRAF containing dimers in KRAS-mutant cancer cell lines; and in the absence of CRAF, ARAF homodimers paradoxically hyperactivate the MAPK pathway leading to excessive and cytotoxic levels of p-ERK. Lastly, ARAF homodimer-inducing mutations were recently reported that confer resistance to the RAF dimer inhibitor belvarafenib. Thus, paralog selectivity may be a critical factor driving RAF dimer inhibitor escape mechanisms and failure to engage all RAF alleles may limit antiproliferative potential depending on the genetic context of RAF and RAS. Accordingly, methods to accurately quantify drug engagement of BRAF, CRAF, and ARAF in the context of the RAS signaling complex represent a critical unmet need in early drug development.
discovery, as cell-free methods that use RAF kinase domain fragments as proxies for the signaling complex are reportedly inaccurate\textsuperscript{20,23}.

We developed a method to systematically quantify drug-target occupancy at selected RAF paralogs within an active RAS-RAF signalosome in live cells. Extending on the basic NanoBRET principle, this method utilizes a conditional BRET reporter system\textsuperscript{24–26} based on an energy transfer donor originating from structural complementation of luciferase reporter subunits, in this case fused to individual RAF protomers\textsuperscript{5,27}. Cell-permeable, ATP-competitive fluorescent probes report on engagement at the luminescent complex in cells, enabling quantitative protomer-selective readouts for BRAF, CRAF, or ARAF in the RAS-RAF signalosome. RAF target engagement can be readily evaluated in the presence of mutant KRAS alleles such as G12C and G13D, which are reported to represent a spectrum of GTP-bound states\textsuperscript{28}. Sensitization of RAF engagement via KRAS-GTP enables the high affinity state of distinct aC-helix stabilizing chemotypes to be observed in an isogenic setting. Moreover, this method reports on the relative selectivity of RAF dimer inhibitors for BRAF/CRAF over BRAF/ARAF heterodimers in the presence of hotspot mutant alleles of KRAS. We profiled a broad cohort of aC-in/DGF-out inhibitors to query their engagement potential at BRAF/CRAF/ARAF heterodimers. Few RAF dimer inhibitors were capable of potently engaging ARAF-containing dimers in cells, and ARAF protomer engagement appears to be a potency determinant in MAPK pathway inhibition in a KRAS mutant settings. We present this suite of cell-based tools to evaluate RAF dimer inhibitors in drug discovery workflows.

**Results**

**Observing the impact of mutant KRAS on wildtype BRAF target engagement**

RAF dimer inhibitors are reported to engage BRAF in the aC-helix-IN conformation, which is also the conformation stabilized by interactions with active RAS. However, engagement of RAF dimer inhibitors to BRAF in complex with RAS-GTP has not been evaluated in live cells. We queried BRAF target engagement of multiple chemotypes in cells under conditions of high vs low KRAS activity to probe the high affinity state of both aC-IN and aC-OUT RAF inhibitors by expressing BRAF-Nanoluc with and without exogenous KRAS (G12C) and employing a cell-permeable BRET probe previously used to measure kinase occupancy in live cells\textsuperscript{25,26} (Fig. 1B). In this configuration, engagement is measured by competitive displacement of the BRET reporter complex between BRAF-NanoLuc, and the fluorescent kinase tracer formed under dynamic equilibrium.

To query engagement of BRAF under low and high KRAS-GTP conditions, the BRET probe must have the capacity for engagement regardless of BRAF activation state. Among a collection of BRET probes evaluated (Supplementary Figs. 1A and 1B), BRET probe 5 (Fig. 1B) was selected owing to its unique ability to engage BRAF under both low and high RAS conditions. In the absence of ectopic KRAS expression, the binding of BRET probe 5 was not saturable up to its solubility limit (1 µM) but the
magnitude of the BRET signal was strong enough to support target engagement assays (0.240 µM $K_d$-apparent and 1.7-fold BRET change at 1 µM BRET probe, Supplementary Fig. 1C). In contrast, co-expression of KRAS (G12C) increased probe affinity and BRET amplitude (0.088 µM $K_d$-apparent and 4.7-fold change at 1 µM BRET probe, Supplementary Fig. 1D). This effect was not unique to the KRAS (G12C) allele. Potentiation of BRET was also observed with BRET probe 5 via coexpression of BRAF-NanoLuc with KRAS (G12D) and (G13D) (Supplementary Fig. 1E). Therefore, this BRET system was exquisitely sensitive to a number of mutant KRAS alleles and served to quantify BRAF engagement for all classes of RAFi under both low and high KRAS states.

In general, BRAF target engagement sensitivity to KRAS was correlated with expected or known $\alpha$-C-helix conformation stabilization (Fig. 1C, Supplementary Fig. 2, and Extended Data Table 1). For example, $\alpha$-C-OUT inhibitors, including PLX-4720 and vemurafenib, as well as the paradox breakers PLX-8394 and PLX-7904, were relatively insensitive to KRAS (G12C), demonstrating a modest 3-to-5-fold enhancement in BRAF affinity in the presence of KRAS G12C. The potencies of dabrafenib and encorafenib were more affected by KRAS (G12C), with 9- and 21-fold shifts in potency, respectively. In contrast, all thirteen $\alpha$-IN chemotypes tested showed pronounced sensitivity to KRAS (G12C) overexpression, consistent with their ability to engage BRAF in mutant KRAS-driven tumors. For two RAF dimer inhibitor examples, LXH254 exhibited a 26-fold shift in engagement with a $K_d$-apparent of 18 nM under high RAS conditions, and belvarafenib had a 39-fold RAS-dependent shift in potency, with a $K_d$-apparent of 31 nM in the presence of KRAS G12C (Fig. 1C and 1D and Extended Data Table 1).

The effect of high KRAS on BRAF target engagement for LXH254 and belvarafenib, was also not unique to the G12C allele. The sensitization to BRAF engagement was reproduced for other alleles such as G13D, that may be even more biased to the GTP state in cells. However, target engagement of BRAF / high-RAS did not agree with the reported potency of MAPK pathway attenuation in either KRAS (G12C) lineages (e.g., Mia PaCa-2 cells, where treatment of LXH254 resulted in a phospho-ERK1/2 (p-ERK) IC$_{50}$ of 687 nM), nor in our own analysis of p-ERK inhibition in a KRAS (G13D)-driven line (DLD1), (LXH254 p-ERK IC$_{50}$ of 150 nM) (Fig. 1E and Extended Data Table 2). Similarly, in a KRAS (G13D) setting, belvarafenib engaged BRAF at much lower concentrations than those attenuating p-ERK levels in DLD1 cells (Fig. 1E and Extended Data Table 2). This discrepancy opens the possibility that BRAF engagement may be necessary, but not a potency-limiting factor in MAPK-pathway attenuation.

**A method to quantify target occupancy at individual RAF protomers within the RAS/RAF complex**

Although our initial target engagement assays revealed the impact of mutant KRAS on BRAF engagement, this design is insufficient to query the occupancy of a compound at individual protomers within RAF homo- or heterodimers. In addition, the measurements obtained in the conventional assay set up provide a global readout of engagement at potentially multiple populations of BRAF. The majority of
expressed BRAF-nanoLuc in the cell is presumed to be homo-dimerized and complexed to mutant KRAS. However, energy transfer could also arise from monomeric BRAF, or BRAF complexed with endogenous CRAF or ARAF proteins. To provide better resolution on the mechanism of action for RAF dimer inhibitors, we designed a target engagement system that conditionally assesses occupancy at individual protomers within RAF dimers in complex with RAS-GTP.

To achieve this goal, we implemented a structural complementation system derived from NanoLuc (NanoBiT). By tagging BRAF and CRAF protomers with either subunit of NanoBiT (SmBit, LgBiT), a direct interaction can be measured in live cells using bioluminescence (Fig. 2A). Using this NanoBiT system, it is possible to observe and quantify the impact of oncogenic mutant alleles of KRAS alone, or in combination with a RAF inhibitor on the stabilization of this signaling complex. High RAS conditions resulted in a pronounced (39-fold) increase in luminescence for BRAF and CRAF dimerization compared to that observed under low RAS conditions (Fig. 2B). As expected, RAFi-mediated dimerization was also observed for specific compounds, which was potentiated under high RAS conditions (Figs. 2B, Supplementary Fig. 3, and Extended Data Table 3). Synergy between RAS and compound-mediated stabilization of RAS/RAF complexes was observed for all αC-IN and αC-OUT RAF inhibitors tested, which was not seen using the paradox breaker PLX-8394 up to concentrations as high as 10 µM, consistent with the expected profile of chemotypes that block RAF dimer formation in cells. In further support of complex formation, membrane-localized RAS/RAF complexes were confirmed in cells using bioluminescence imaging (Fig. 2C and Supplementary Fig. 4). MAPK14-NanoLuc served as a control for cytosolic localization, and membrane localized DDR1-NanoLuc illustrated membrane localization, consistent with their expected localization patterns.

We next addressed compound occupancy of individual protomers within the BRAF/CRAF heterodimer complex. The combination of NanoBiT fusions of BRAF-SmBiT and CRAF-LgBiT generated donor luminescence that yielded robust energy transfer. However, protomer selectivity needed to be established since most BRET probes are derived from promiscuous kinase inhibitors. We tested multiple BRET probes using the previously described individual NanoLuc fusions to BRAF and CRAF (Supplementary Fig. 1B and Supplementary Fig. 5A, respectively), and found that while many probes yielded a specific BRET signal with BRAF-NanoLuc, only BRET Probe 6 (Fig. 2D) yielded CRAF-Nanoluc BRET signal under high RAS conditions. Therefore, although BRET Probe 5 could be used to selectively report on the BRAF protomer within the BRAF/CRAF heterodimer, we were not able to identify a BRET probe that would selectively report on the CRAF protomer.

In the absence of a CRAF-selective chemical probe, BRET protomer specificity required kinase domain mutagenesis to prevent tracer binding while simultaneously preserving RAF dimerization capacity. To achieve these two functions, we took advantage of previously reported BRAF(A481F) and CRAF(A373F) active site mutants that block ATP-binding but create a pseudokinase where the scaffolding functions of the RAFs are preserved (Figs. 2E and 2F). Introduction of these mutations to the BRAF-NanoLuc and CRAF-NanoLuc constructs successfully prevented BRET probe binding under high RAS conditions (Supplementary Fig. 5B). However, when incorporated into the NanoBiT constructs, BRAF and CRAF
possessing these mutations retained the capacity to dimerize (Supplementary Fig. 6A and 6B). In BRET experiments, the BRAF/CRAF heterodimer demonstrated large BRET signals with both BRET Probes 5 and 6, as expected (Supplementary Fig. 6C and 6D). Dimers containing both BRAF(A481F) and CRAF(A373F) did not show BRET with either Probe, confirming that the mutations still prevent probe binding in the dimeric context. Heterodimers between wildtype BRAF and CRAF(A373F) still demonstrated BRET with both BRET probes 5 and 6, consistent with the ability of BRAF to bind both probes in isolation, while heterodimers containing BRAF(A481F) and wildtype CRAF only produced BRET signal with BRET Probe 6, but not BRET Probe 5, consistent with our results from tracer binding experiments to CRAF in isolation. For simplicity, we chose to move forward with BRET probe 6 for analysis of engagement at either the BRAF or CRAF protomers when paired with the opposite mutated RAF protomer (Figs. 2E and 2F).

To extend this assay approach to evaluate RAF dimer inhibitor selectivity for ARAF, we screened the panel of BRET probes against both ARAF-NanoLuc fusions in isolation and against BRAF(A481F)/ARAF heterodimers under KRAS (G12C) conditions. Though none of the BRET probes demonstrated sufficient BRET with the isolated ARAF-NanoLuc fusions under high or low RAS conditions (Supplementary Figs. 7A and 7B), BRAF(A481F)/ARAF heterodimers demonstrated a strong BRET signal with BRET Probe 5 (Supplementary Fig. 7C). These results suggest that the ARAF active site conformation in isolation is pharmacologically distinct compared to its form in heterodimers and provide a clear strategy for selectively measuring engagement at the ARAF protomer within BRAF(A481F)/ARAF heterodimers using BRET Probe 5 (Fig. 2G). To confirm that we could evaluate pan-RAF protomer engagement in the presence of other mutant KRAS alleles, we confirmed that all three protomer engagement assays were functional using KRAS(G13D), which yielded similar results to G12C (Supplementary Fig. 8).

Paralog selectivity in cells: Engagement of ARAF protomers is commensurate with MAPK-pathway inhibition in mutant RAS-driven lineages.

Given the importance of establishing engagement symmetry at dimers comprised of BRAF and CRAF and the emergence of ARAF-containing dimers as a novel target in RAS-driven cancers, we used our newly developed suite of dimeric RAF protomer assays to assess the RAF paralog selectivity profile of various type II RAF dimer inhibitors in the presence of KRAS (G12C), including a number of candidates undergoing clinical trials (Fig. 3A and Supplementary Fig. 9). Subsequently, the engagement potencies were compared to calculate a selectivity ratio between BRAF and either CRAF or ARAF protomers (Extended Data Tables 4 and 5). In a KRAS (G12C) setting, all thirteen compounds tested demonstrated potent and nearly symmetrical RAF binding of BRAF and CRAF protomers with a slight preference for CRAF, as evidenced by CRAF/BRAF selectivity ratios < 1, confirming the intended mechanism of action of these inhibitors to occupy both BRAF and CRAF protomers within RAF heterodimers over the same drug concentration range. LXH254 was found to be the most potent inhibitor in cells, with IC$_{50}$s of 6.2 nM and 4.0 nM for BRAF and CRAF protomers, respectively, followed closely by LY3009120, RAF709, TAK-632, and belvarafenib. In contrast, RAF265 was found to be the weakest inhibitor with IC$_{50}$s of 530 nM and 180 nM for the BRAF and CRAF protomers, respectively.
In contrast to the consistent CRAF/BRAF selectivity indices of all inhibitors tested (range 0.2 – 0.8-fold), the ARAF/BRAF selectivity indices varied widely (9 – 75-fold) and were significantly biased toward BRAF protomer engagement, indicating that the majority of αC-helix-IN stabilizing drugs asymmetrically engage ARAF-containing heterodimers in cells (Fig. 3A, Supplementary Fig. 9, and Extended Data Tables 4 and 5). Among the thirteen compounds studied in the KRAS (G12C) background, lirafenib demonstrated the greatest potential for concurrent protomer engagement within ARAF heterodimers (ARAF/BRAF ratio = 9) and AZ-628 and LXH254 demonstrated the weakest engagement of ARAF protomers compared to BRAF protomers (ARAF/BRAF ratios of 75 and 53, respectively). In terms of absolute potency, LY3009120 and RAF709 were the most potent ARAF inhibitors with IC$_{50}$s of 200 nM and 220 nM, respectively. In contrast, RAF265 and TAK-580 were the weakest ARAF inhibitors in the set, with IC$_{50}$s of 6400 nM and 4000 nM, respectively. This pronounced offset in ARAF protomer engagement was not unique to a KRAS (G12C) background and was also observed in the presence of KRAS (G13D) (Fig. 3B, Extended data table 5). Notably, purified enzymatic assays did not demonstrate such ARAF avoidance, and supported pan-RAF inhibitor potential (i.e., equal potency among all three RAF paralogs) (Fig. 3C, Supplementary Fig. 10, and Extended Data Table 6). Overall, these results demonstrate that the compounds tested are effectively dual RAF inhibitors for BRAF/CRAF heterodimers but are markedly weak against ARAF protomers within ARAF/BRAF heterodimers.

The apparent affinities for LXH254 to BRAF and CRAF protomers in cells at KRAS (G12C) complexes were nearly 100-fold stronger than the potency (687 nM) previously reported for MAPK-pathway inhibition in MiaPaCa-2 cells (KRAS G12C)$^{20}$. In contrast, engagement of ARAF protomers at KRAS (G12C) complexes with LXH254 agreed closely with inhibition of p-ERK$^{20}$. The agreement between ARAF engagement and MAPK pathway inhibition was not unique to the KRAS (G12C) setting and was also observed for KRAS(G13D). ARAF protomer engagement of LXH254, belvarafenib, and LY3009120 in BRAF heterodimers at KRAS (G13D) complexes was commensurate with p-ERK attenuation in DLD-1 cells (KRAS(G13D)), whereas BRAF and CRAF protomer engagement was prominently left-shifted (Fig. 3D, Supplementary Fig. 11, and Extended Data table 2). These results maintain that inhibition of BRAF/CRAF heterodimers may be necessary, but insufficient to inhibit MAPK signaling in certain genetic backgrounds and demonstrate the need for deeper characterization of RAF dimer inhibitors at ARAF-containing heterodimers in cells.

**Discussion**

In mutant KRAS-driven cancers, the therapeutic utility of RAF inhibitors is limited by their capacity to concurrently occupy both RAF protomers within the RAS-RAF signalosome. Dual protomer engagement is also critical when treating BRAF-mutant cancers, to combat drug resistance due to feedback amplification of the canonical MAPK pathway and upregulation of upstream signaling. However, assessments of drug affinity for individual RAF proteins have largely been limited to purified kinase domains. Moreover, RAF binding and/or inhibition is typically measured in the absence of RAS, failing to provide a context in which to observe the high affinity state of RAF dimer inhibitors. Since engagement
for RAF dimer inhibitors is driven by intracellular levels of RAS-GTP, the genetic context of RAS may dictate the vulnerability of various tumor cell types to wildtype RAF engagement. Therefore, it is not surprising that cell-free/biochemical assessments using kinase domain fragments are often inaccurate and fail to correlate with known biomarkers of MAPK activity measured in cells.

Here we directly assessed the impact of mutant KRAS on target occupancy of RAF inhibitors. In BRAF target engagement, RAF dimer inhibitors were the class of drugs most sensitive to overexpression of mutant KRAS (both G12C and G13D), consistent with their structural propensity to stabilize the kinase αC-helix-IN conformation. As described, the range of KRAS(G12C)-dependent IC_{50} shifts for these inhibitors was 15–71-fold. In contrast, KRAS (G12C) imparted a relatively weak influence on the intracellular engagement for αC-OUT inhibitors, apart from dabrafenib and encorafenib. This phenomenon appears to be related to the degree by which the αC-helix is held between a fully αC-IN or fully αC-out conformation. While classified as "αC-out," the x-ray crystal structure of dabrafenib bound to BRAF V600E shows the αC-helix is only in a partially "OUT" conformation, halfway between the orientation of the αC-helix in structures of PLX-7904 or vemurafenib compared to LY3009120 (Figure 4). Correspondingly, the potency of dabrafenib engagement was left-shifted under high KRAS conditions by 9-fold, a value that is in between the range of shifts observed for fully αC-OUT and αC-IN compounds. There is no solid-state structure of encorafenib bound to BRAF, but we suspect the αC-helix is oriented in a similar fashion to dabrafenib based on these observations. The spectrum of sensitization by KRAS (G12C) for these varied chemotypes suggests that a binary categorization of RAF inhibitors by αC-helix-IN and OUT may be insufficient. Predicting the vulnerability of RAF-engagement in a mutant KRAS context may be difficult based solely on structural or enzymatic studies with pure protein. By querying engagement under low and high RAS states, this method makes it possible to infer subtle structural features of such inhibitors based on their biochemical properties in cells. Moreover, this method makes it possible to prioritize the intracellular potency of various RAFi against BRAF in the presence of any mutant KRAS allele of interest, which should be enabling in the pursuit of new medicines targeting RAS-driven cancer.

Despite the ability to query the RAS-driven, high affinity state of RAF dimer inhibitors at BRAF, target engagement was orders of magnitude stronger than inhibition of MAPK signaling in multiple RAS-driven lineages. This underscores the fact that engagement of BRAF alone is insufficient to inhibit MAPK signaling in the presence of active RAS and validates the need for evaluation of protomer engagement for the remaining RAF paralogs. To query occupancy of selected RAF protomers, we extended the utility of the basic NanoBRET Target Engagement method. This iteration is based on a BRET donor conditionally originating from protein dimers, enabling protomer-selective readouts for BRAF, CRAF, or ARAF within a RAS/RAF signalosome. Current methods query engagement to a population of targets, regardless of interaction status. The ability to measure compound displacement of a signal that is generated from a single condition (i.e., one protomer within RAS-RAF dimers – and only while dimerized), rather than
multiple states of the target of interest, provides a precise interpretation of compound pharmacology in cells.

The NanoBiT assay enabled us to quantify RAFi engagement of ARAF within RAF heterodimers, which may be a bona fide therapeutic target in RAS-driven cancers. Evaluation of a broad panel of RAF dimer inhibitors revealed that avoidance of the ARAF protomer within these heterodimers is a general property of type II RAFi. Strong engagement BRAF and CRAF protomers, and avoidance of ARAF was observed under both high KRAS (G12C) and KRAS (G13D) conditions. Moreover, ARAF engagement closely agreed with inhibition of p-ERK in DLD-1 (G13D) cells and MIA PaCa-2 (G12C) cells, supporting the notion that ARAF engagement is a potency-limiting limiting factor in these cells. It is noteworthy that cell-free, biochemical analyses were incapable of recapitulating the intracellular paralog selectivity for such compounds. Enzymatic IC$_{50}$s with purified BRAF, CRAF, and ARAF kinase domains were not in agreement with the intracellular BRET results, as these assays reported equipotent inhibition of BRAF and ARAF (IC$_{50}$s within 2-fold). This may be due to the use of monomeric kinase domains in the absence of RAS-GTP complexation. Indeed, during the development of ARAF-protomer assay we found that BRET probes only bound ARAF in the context of BRAF heterodimers, failing to generate BRET from that ARAF-Nanoluc monomers and homodimers.

Avoidance of ARAF may represent an opportunity to achieve improved therapeutic index in some normal cells and tissues, but the failure to engage such dimers is also a clear liability when aiming to deplete cells of MAPK signaling in certain cancers. Extending the lessons learned from paradoxical activation of wildtype BRAF in the presence of V600E-inhibitors, compounds with asymmetric engagement of heterodimers could possibly trigger paradoxical activation of RAF signaling in RAS-mutant cells, or contribute to drug-resistance in mutant BRAF-driven cancers that escape initial therapy$^{20}$. Our results indicate that increase focus should be placed on development of true pan-RAF inhibitors that concurrently engage all RAF paralogs within such complexes.

While cell-free kinase assays are convenient and often enabling in a high-throughput screening environment, they may fail to predict selectivity in cells. Furthermore, in vivo drug pharmacology may be driven by dynamic protein complexes that are challenging to simulate in a cell-free system, as evidenced here using the RAS/RAF signalosome as an example. To our knowledge this represents the first method uniquely capable of exquisitely conditional analysis at hetero-multimeric complexes in live cells. We intend the RAS/RAF system described here as a template for a new platform to quantify drug engagement within select protein complexes in live cells, that should be adaptable to other model systems. As the scaffolding functions of kinases within dynamic multiprotein complexes are more fully
elucidated, this method will be applicable to a variety of targets, providing new insights into these signaling complexes and a better mechanistic understanding of drug action in vivo.

Methods

Experimental Models and Subject Details

HEK-293 cells (ATCC) were cultured in DMEM (Gibco) + 10% FBS (Seradigm), with incubation in a humidified, 37°C/5% CO₂ incubator. DLD-1 cells (ATCC) were cultured in RPMI-1640 (GIBCO, cat. 21875091) plus 10% FBS (GIBCO, cat. 10270106), 2 mM L-glutamine (GIBCO, cat. 25030024), 100U penicillin, and 0.1 mg/mL streptomycin.

BRET Probes and Chemical Inhibitors

All chemical inhibitors were from common vendors such as Selleckchem and Med Chem Express. BRET probes 1 and 6 were synthesized as described previously \(^25\). BRET probes 3, 4, and 5 were synthesized as described previously \(^33\).

Cell Transfections and BRET measurements for RAF/NanoLuc fusions under high and low RAS conditions.

For RAF cellular BRET measurements using NanoLuc fusions, N- or C-terminal NanoLuc fusions to the RAFS were encoded in pFN31K or pFC32K (respectively) expression vectors (Promega), including flexible GSSG linkers in between the tag and each RAF. The ARAF, BRAF, and CRAF ORFs were full length and based upon UniProt isoform 1 (P10398-1, P15056-1, and P04049-1, respectively). All KRAS ORFs were full length and based upon KRAS4B (UniProt P01116-2). All transfections used FuGENE HD (Promega) according to the manufacturer’s protocol. For conditions defined as “low RAS” in HEK-293 cells, RAF/NanoLuc fusion constructs were diluted into Transfection Carrier DNA (Promega) at a mass ratio of 1:9 (mass/mass) in Opti-MEM (Gibco), after which FuGENE HD was added at a ratio of 1:3 (mg DNA: µL FuGENE HD). For example, for a 1mL size transfection complex, 1µg each of the RAF/NanoLuc fusion DNA was combined with 9µg Transfection Carrier DNA in 1 mL of Opti-MEM. 1 part (vol) of FuGENE HD complexes thus formed were combined with 20 parts (vol) of HEK-293 cells suspended at a density of 2 x 10^5 per mL in Opti-MEM containing 1% (v/v) FBS. The cell suspensions were seeded at 100uL per well into white tissue culture treated assay plates (Corning CAT#3917), followed by incubation in a humidified, 37°C/5% CO₂ incubator for 18 – 24 hr. The total amount for each RAF plasmid was 5 ng/well, and the total amount of DNA was 50 ng/well. For conditions defined as “high RAS” in HEK-293 cells, the transfection conditions were identical except that the Transfection Carrier DNA was replaced by an expression vector encoding untagged KRAS(G12C) or another KRAS mutant where indicated.

All chemical inhibitors were prepared as concentrated stock solutions in DMSO (Sigma-Aldrich) and diluted in Opti-MEM (unless otherwise noted) to prepare working stocks. Cells were equilibrated with BRET probes and test compound prior to BRET measurements, with an equilibration time of 2 hours.
unless otherwise noted. BRET probes were prepared first at a stock concentration of 100X in DMSO, after which the 100X stock was diluted to a working concentration of 20X in BRET probe dilution buffer (12.5 mM HEPES, 31.25% PEG-400, pH 7.5). For BRET probe dose response measurements, the BRET probes were added to the cells in an 8 point, 2-fold dilution series starting at a final concentration of 1µM. For target engagement analysis, BRET probe 5 was added to the cells at a final concentration of 1µM for low RAS conditions or 0.031µM for high RAS conditions. To measure BRET, NanoBRET Target Engagement Substrate (Promega) and Extracellular NanoLuc Inhibitor were added according to the manufacturer's recommended protocol, and filtered luminescence was measured on a GloMax Discover luminometer equipped with 450 nm BP filter (donor) and 600 nm LP filter (acceptor), using 0.5 s integration time. Raw BRET ratios were calculated by dividing the acceptor counts by the donor counts. Milli-BRET units (mBU) were calculated by multiplying the raw BRET values by 1000. When normalized BRET was used, mBRET values were normalized using Eq. 1;

\[
\text{Normalized BRET (\%) = } \frac{A-C}{B-C} \times 100
\]

Where A = mBRET in the presence of test compound and BRET probe, B = mBRET in the presence of vehicle and BRET probe, and C = mBRET in the presence of a saturating dose of test compound (20µM LY-3009120 unless otherwise noted). Apparent BRET probe affinity values (EC\(_{50}\)) were determined using the sigmoidal dose-response (variable slope) equation available in GraphPad Prism (Eq. 2);

\[
Y = Bottom + \frac{(Top-Bottom)}{\left(1 + 10^{\left((\log EC_{50}-X) \times HillSlope\right)}\right)}.
\]

For determination of test compound potency, competitive displacement data were plotted with GraphPad Prism software and data were fit to Eq. 2 to determine the IC\(_{50}\) value.

**BRET measurements for individual protomers within RAF dimers**

For cellular BRET measurements of RAF protomer target engagement a luciferase donor signal was produced at mutant KRAS-induced RAF heterodimers using a NanoBiT approach. N-terminal SmBiT fusions were encoded in pNB4K expression vectors, including flexible 15 residue linkers (GSSGGGGSGGGGSGG) between the tag and each RAF. C-terminal LgBiT and SmBiT fusions were encoded in pNB1K and pNB2K vectors, respectively, including flexible 15 residue linkers (GSSGGGGSGGGGSGG) between the tag and each RAF. All RAF and KRAS ORFs were based upon UniProt isoforms as described in the previous section. Transfections were performed using FuGENE HD as described in the previous section. Transfection ratios for assays of BRAF/CRAF heterodimers utilized 1 part of each NanoBiT-RAF fusion combined with 38 parts untagged KRAS mutant expression vector or where indicated. For example, for a 1mL size transfection complex, 0.25µg each of the RAF/NanoBiT fusion DNA was combined with 9.5µg KRAS(G12C) expression vector in 1 mL of Opti-MEM. Transfection ratios for assays of BRAF/ARAF heterodimers utilized 1 part of each NanoBiT-RAF fusion combined with 8 parts untagged KRAS mutant expression vector. For example, for a 1mL size transfection complex, 1µg
each of the RAF/NanoBiT fusion DNAs was combined with 8µg KRAS(G12C) expression vector in 1 mL of Opti-MEM. BRET assays were performed as described in the previous section, except that BRET Probe 6 was used at a final concentration of 1µM for assays of the BRAF protomer and CRAF protomer within BRAF/CRAF(A373F) and BRAF(A481F)/CRAF heterodimers, respectively. BRET Probe 5 was used at a final concentration of 0.13µM for assays of the ARAF protomer within BRAF(A481F)/ARAF heterodimers. To measure BRET, the NanoBRET Target Engagement Substrate (Promega) was added according to the manufacturer’s protocol, and luminescence was measured as described in the previous section. All treatment of data and determination of BRET probe and test compound affinity was as described in the previous section.

**Measurements of PPI induction for BRAF/CRAF heterodimers in cells using NanoBiT**

Mutant KRAS and/or compound induced BRAF/CRAF heterodimerization was determined in HEK-293 cells using a NanoBiT approach, utilizing the BRAF-SmBiT, CRAF-LgBiT, and untagged KRAS(G12C) expressed vectors described in the previous sections. Transfections were performed using FuGENE HD as described in the previous section. Transfection ratios for assays of BRAF/CRAF heterodimers utilized 1 part of each NanoBiT/RAF fusion combined with 198 parts of either Transfection Carrier DNA (low RAS) or KRAS(G12C) expression vector (high RAS). For example, for a 1mL size transfection complex, 0.05µg each of the BRAF-SmBiT and CRAF-LgBiT fusion DNA was combined with 9.9µg Transfection Carrier DNA or KRAS(G12C) expression vector in 1 mL of Opti-MEM. 1 part (vol) of FuGENE HD complexes thus formed were combined with 20 parts (vol) of HEK-293 cells suspended at a density of 2 x 10^5 per mL in Opti-MEM containing 1% (v/v) FBS. The cell suspensions were seeded at 100µL per well into white tissue culture treated assay plates (Corning CAT#3917), followed by incubation in a humidified, 37°C/5% CO₂ incubator for 18 – 24 hr. Test compound dilution series (4-fold) or vehicle were prepared at a 1000X concentration in pure DMSO and then diluted to a 10X working concentration in Opti-MEM. 10X Compounds were added to cells and equilibrated for 2 hours at 37°C in a 5% CO₂ incubator unless otherwise noted. After equilibration, the cells were removed from the incubator and allowed to re-equilibrate to room temperature for 15 minutes before adding NanoBRET Target Engagement Substrate. Unfiltered luminescence was measured using the Nano-Glo protocol on a Glomax Discover luminometer with a 0.3 s integration time.

**Measurements of Total LgBiT fusion levels**

Total LgBiT expression level was determined in the presence of a saturating concentration (100nM) of high affinity HiBiT peptide (Peptide 2.0), 1X NanoBRET Target Engagement substrate, and 50 µg/mL digitonin (as a permeabilization agent). Unfiltered luminescence was measured using the Nano-Glo protocol on a Glomax Discover luminometer with a 0.3 s integration time.

**Bioluminescent Imaging of NanoBiT-KRAS fusions in live cells**
HEK-293 cells were harvested and resuspended to a density of 5x10^4 cells/mL in growth media (DMEM + 10% FBS). Cells were plated onto Nunc Lab-Tek II 8-well chambered coverslips (Thermo Fisher) coated with 0.1% gelatin in 400 µl growth medium (DMEM + 10% FBS) at a density of 2x10^4 cells per well and incubated at 37°C overnight. The following day, growth media was aspirated and replaced with 400uL OptiMEM + 4% FBS, and transfection complexes were set up using FuGENE HD and plain OptiMEM as described above. Transfection ratios for assays of BRAF/CRAF NanoBiT heterodimers were as described for measuring BRET at individual RAF protomers (1 part of each NanoBiT-RAF fusion combined with 38 parts untagged KRAS(G12C)). Control plasmids encoding MAPK14-NanoLuc and DDR1-NanoLuc fusions were from Promega and were diluted into Transfection Carrier DNA (Promega) at a mass ratio of 1:9 (mass/mass) in Opti-MEM. Transfection complex was added to plated cells at a ratio of 1:20 (v/v), as previously described. After 24 h of incubation at 37°C, 200 µl of Nano-Glo Live Cell Reagent (Promega) was added immediately prior to imaging. All imaging experiments were performed using the LV200 bioluminescence imaging system (Olympus) equipped with an ImagEM X2 EM-CCD camera (Hamamatsu) and a 40x Oil, 1.4 NA objective. Images were acquired with cellSens software (Olympus) using electron multiplying (EM) gain and exposure time settings as listed in the source data table. Each image was generated using an average projection of 10 images. Generation of average projections and linear adjustments of dynamic range were performed using Python and Image J image processing software (Fiji package).

**Determination of Cell Free RAF Inhibition Potency for RAFi**

Cell free IC_{50}s of RAFi were performed at Nanosyn (Sunnyvale, CA) using a cascade MAPK-pathway assay format, measuring the ultimate phosphorylation of a peptide substrate for ERK. Compounds (250 nl of a 100x DMSO stock) were dispensed by an Echo 650 acoustic liquid handler (Beckman) into a 384-well microtiter plate and mixed with an initial volume of 5 µl of ARAF (BPS, cat. 40010, lot 130118-G, 18 nM), BRAF (Millipore, cat. 14–530, lot 203205-A, 6 nM), or CRAF (BPS, cat. 40008, lot 200501-G1, 0.9 nM) in 100 mM HEPES kinase reaction buffer, pH 7.5, containing BSA (0.1%, Sigma, cat. A3059), Triton X-100 (0.01%), DTT (1 mM), MgCl_2 (5 mM), sodium orthovanadate (10 µM), and beta-glycerophosphate (10 µM). No enzyme and DMSO only served as positive and negative controls, respectively. After a 30-minute preincubation at room temperature, an additional 10 µl of kinase reaction buffer containing inactive MEK1 (Millipore, cat. 14–420, lot 2350988-M, 7.5 nM), inactive ERK2 (ThermoFisher, cat. PV3314, lot 1760987K, 3 nM), and an initial low concentration of ATP (0.3 µM for ARAF and BRAF, 0.75 µM for CRAF, Sigma, A7699) were added to each well and incubated for 1 h at 25 ºC. The final “RAF-reaction” volume was 15 µl with 1.67% DMSO. A custom ERK peptide substrate conjugated to carboxyfluorescein (Nanosyn, 1 µM) in 10 µl kinase buffer containing high ATP (62.5 µM) and staurosporine (0.25 µM) was then added to each well and incubated for another 2 h at room temperature. Reactions were terminated with 40 µl EDTA-containing buffer. Substrate and product were separated electrophoretically using the microfluidic-based LabChip 3000 Drug Discovery System (Caliper Life Sciences) and quantitated by fluorescence intensity. Percent inhibition of product formation (AUC of product peak) at each dose was calculated using Eq. 3:
\[
\text{Inhibition (\%)} = \frac{(S-NC)}{(PC-NC)} \times 100.
\]

Where sample data (S) were normalized between 0 and 100% inhibition (NC = negative control and PC = positive control, respectively). Potency values (IC\(_{50}\)) were determined using the sigmoidal dose-response (variable slope) equation available in GraphPad Prism (Eq. 2).

**Determination of Phospho-ERK Inhibition Potency for RAFl**

TR-FRET measurements of phospho-ERK were conducted by IRBM (Rome, Italy, SOP 941.321) using the Advanced Phospho-ERK1/2 (Thr202/Tyr204) kit from CisBio (cat. 64AERPET), according to the manufacturer’s protocols. Briefly, DLD-1 cells were seeded using a TEMPEST® liquid handler (Formulatrix) in 384-well, white-bottom assay plates (Corning, cat. 784080) at 5,000 cells/12 µl per well. After 24 h incubation at 37°C + 5% CO\(_2\), compounds were dispensed by an Echo 650 acoustic liquid handler (Beckman) (compound stocks were prepared at 1 mM DMSO). Trametinib (5 µM) and DMSO served as positive and negative controls, respectively, and the final plated DMSO concentration was 0.5% across all conditions. After a 2 h compound incubation at 37°C, the lysis and blocking buffer mix (4 µl/well) was dispensed and incubated for 1 h at room temperature on a plate shaker, followed by addition of the antibodies mix (4 µl/well). After a 16 h incubation at 4°C in the dark, the TR-FRET signal was measured using an EnVision® plate reader (PerkinElmer) with the following parameters: cycle 10000, delay 100, number of flashes for each detector = 20, number of sequence windows = 1, total time of windows = 500. Samples were excited at 320 nm; the reading from channel 1 was recorded at 665 nm, and the reading from channel 2 was recorded at 615 nm. Envision data for channel1 and channel2 were imported into Dotmatics and pre-processed by calculating the channel1/channel2 ratio. Sample data (S) were analyzed as normalized between 0 and 100% inhibition (NC = negative control and PC = positive control, respectively) using Eq. 3. For IC\(_{50}\) determinations, the dose-response was fitted with Eq. 2.

**DATA AVAILABILITY**

The authors declare that the data supporting the findings of this study are available within the article, the accompanying Source Data, the Supplementary Information, and the Supplementary Data. Additional information, resources, and reagents will be made available upon reasonable request; requests should be directed to and will be fulfilled by the Lead Contact Matthew B. Robers. Matt.Robers@promega.com

**References**


Figures
Figure 1

**BRAF TE for RAFi under low and high RAS conditions.** (A) Alignment of BRAF co-crystal structures depicting conformations of the αC helix bound to two compounds (LXH254 and PLX-7904). (B) Schematic overview of the target engagement assay and structure of BRET probe 5. BRAF-NanoLuc is co-expressed along with a promoterless carrier DNA (low RAS) or untagged KRAS mutant (high RAS) as indicated in HEK293 cells. The cells are treated with BRET Probe 5 and RAFi and competition of the BRET signal is measured after a 2-hour incubation. (C) BRAF target engagement potency for a panel of 22 RAFi under low and high RAS conditions using KRAS(G12C). For RAF265, data are the mean ± S.E.M from 5 independent experiments (n=5), each collected with 4 technical replicates. For regorafenib, data are the mean ± S.E.M from 4 independent experiments (n=4), each collected with 4 technical replicates. For all
other RAFi, data are the mean ± S.E.M from 3 independent experiments (n=3), each collected with 4 technical replicates. (D) Representative target engagement competition curves under low and high RAS conditions with KRAS(G12C) for LXH254 and belvarafenib. Data are the mean ± S.E.M. from 3 independent experiments (n=3). (E) Comparison of BRAF target engagement potency of LXH254 and belvarafenib under high RAS conditions with KRAS(G13D) to inhibition of phospho-ERK activity in KRAS(G13D) driven DLD1 cells. For BRAF target engagement, data are the mean ± S.D. of 4 technical replicates from a single experiment (n=1). For phospo-ERK, data points are the mean of technical duplicates from a single experiment (n=1).
Figure 2

Conditional target engagement assays for individual protomers within defined RAF heterodimers. (A) Schematic overview of mutant KRAS driven RAF dimer system. BRAF-SmBiT and CRAF-LgBiT are co-expressed along with untagged KRAS(G12C) or a promoterless carrier DNA (low RAS) in HEK293 cells. Luminescence is measured under high or low KRAS(G12C) conditions or in the presence of RAF inhibitor. (B) High KRAS(G12C) increased the degree of BRAF:CRAF dimerization relative to low KRAS(G12C) conditions (39-fold increase in luminescence relative to low RAS conditions). The addition of LXH254 (1.25µM) under high RAS conditions further increased the luminescence (150-fold relative to low RAS conditions and 3.8-fold relative to high Ras conditions). Data are the mean ± S.E.M. of three independent experiments (n=3). (C) Bioluminescence imaging in HEK293 cells of BRAF/CRAF heterodimer localization under high KRAS(G12C) conditions compared to MAPK14-NanoLuc (cytoplasm) and DDR1-NanoLuc (membrane) control constructs. Data are representative of biological duplicates (n=2). (D) Chemical structures of BRET probes used to develop the RAF protomer assays. (E-G) Schematic overview of the RAF protomer assays and impact of BRET probe concentration on engagement potency. RAF/NanoBiT fusions (and mutants thereof) were co-expressed in HEK293 cells along with untagged KRAS(G12C) as described in the methods. The cells are treated with BRET Probe 5 (ARAF protomer) or BRET probe 6 (BRAF and CRAF protomers), and RAFi, and competition of the BRET signal is measured after a 2-hour incubation. Data points for the LY3009120 dilution series at varying BRET probe concentration are the mean ± S.E.M of biological triplicates (n=3). BRET probe concentrations chosen for subsequent IC₅₀ measurements are depicted in solid black.
RAFi target engagement for individual RAF protomers within heterodimers and correlation to phospho-ERK inhibition. (A) Target engagement potency for each RAF protomer under high KRAS(G12C) conditions for various RAFi. For all RAFi, Data are the mean ± S.E.M. of 3 independent experiments (n=3), each collected with 4 technical replicates. (B) Representative RAF protomer target engagement competition curves for LXH254, Belvarafenib, and LY3009120 under high KRAS(G13D) conditions. Data are the mean ± S.E.M. of 3 (ARAF) or 4 (BRAF and CRAF) independent experiments (n = 3−4) each collected with 4 technical replicates. (C) The ARAF vs BRAF protomer selectivity ratio for select RAFi under live cell and cell free assay conditions. The live cell target engagement potencies are from panel B. The cell free IC$_{50}$ values were determined from inhibitor dose responses (n = 3 independent experiments)
measured for recombinant cascade ARAF or BRAF enzyme activity assay formats using a microfluidic-based LabChip 3000 Drug Discovery System (assays performed by Nanosyn, Sunnyvale, CA; reference Extended Data Table 6). (D) Comparison of target engagement potency to potency of phospho-ERK inhibition for select RAFi. RAF protomer target engagement potencies were from panel B. DLD1 cell Phospho-ERK inhibition data was the mean of technical duplicates from a single experiment (n = 1).

Figure 4

Dabrafenib stabilizes an αC-helix conformational state in BRAF that is part-way between fully αC-helix-IN and OUT. Overlays of the two protomers in the x-ray dimer pair for each BRAF structure bound to the indicated inhibitor show the difference in the position of the αC-helix that is stabilized by each inhibitor interaction. This figure is recreated with permission from: Karoulia and coworkers32. PDB accession numbers for BRAF V600E structures left to right: 4XV1, 3OG7, 4XV2, 5C9C.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- RAFExtendedDataTablesVasta17Oct2022.xlsx
- RAFdimerTEpaperSupplementaryVasta17Oct2022.docx
- MRobersEPCflat.pdf