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# New perspectives for targeting RAF kinase in human cancer

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## Abstract

The discovery that a subset of human tumours is dependent on mutationally deregulated BRAF kinase intensified the development of RAF inhibitors to be used as potential therapeutics. The US Food and Drug Administration (FDA)-approved second-generation RAF inhibitors vemurafenib and dabrafenib have elicited remarkable responses and improved survival of patients with BRAF-V600E/K melanoma, but their effectiveness is limited by resistance. Beyond melanoma, current clinical RAF inhibitors show modest efficacy when used for colorectal and thyroid BRAF-V600E tumours or for tumours harbouring BRAF alterations other than the V600 mutation. Accumulated experimental and clinical evidence indicates that the complex biochemical mechanisms of RAF kinase signalling account both for the effectiveness of RAF inhibitors and for the various mechanisms of tumour resistance to them. Recently, a number of next-generation RAF inhibitors, with diverse structural and biochemical properties, have entered preclinical and clinical development. In this Review, we discuss the current understanding of RAF kinase regulation, mechanisms of inhibitor action and related clinical resistance to these drugs. The recent elucidation of critical structural and biochemical aspects of RAF inhibitor action, combined with the availability of a number of structurally diverse RAF inhibitors currently in preclinical and clinical development, will enable the design of more effective RAF inhibitors and RAF-inhibitor-based therapeutic strategies, tailored to different clinical contexts.

The family of RAF kinases (ARAF, BRAF and CRAF (also known as RAF1)) constitute core components of the RAS–RAF–MEK–ERK signalling cascade (ERK signalling), a pathway that mediates signals from cell surface receptors to the nucleus to regulate cell growth, differentiation and survival[1-4]. Upon activation, RAF kinases phosphorylate and activate the kinases MEK1 and MEK2, which in turn phosphorylate and activate ERK1 and

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**Author contributions**

Z.K., E.G. and P.I.P. contributed equally to researching the literature, to writing the article and to reviewing and editing the manuscript before submission.

**Competing interests statement**

The authors declare no competing interests.

**SUPPLEMENTARY INFORMATION**

See online article: S1 (movie) | S2 (movie)

ERK2. Activated ERK promotes cell proliferation and survival by phosphorylating multiple substrates both in the cytosol and in the nucleus[4].

Deregulation of ERK signalling commonly occurs in cancer, frequently due to mutations of components of the pathway[5]. It has been known for more than four decades that mutations in the genes encoding for the RAS family of proteins are often found in human tumours[6,7]. RAS protein has been extremely difficult to target, and thus the downstream kinases RAF, MEK and ERK remain attractive therapeutic targets in such tumours, although current inhibitors of these kinases have only showed limited efficacy in RAS-mutant cancers[8]. *BRAF* mutations are present in approximately 8% of human tumours[9]. The most frequent mutation in the *BRAF* gene is the 1799T>A substitution that results in an amino acid change from valine (V) to glutamic acid (E) in the activation segment of the kinase (V600E), promoting several-fold kinase hyperactivation[9,10]. In addition to V600E, and other substitutions at V600 (such as V600K, V600D and V600R), a number of non-V600 missense *BRAF* mutations have been found, mostly clustered in the activation segment or in the so-called glycine-rich loop of the kinase domain[9-11]. Point mutations are not the only alterations found in *BRAF*. Fusion proteins resulting from translocations containing the catalytic domain of BRAF, as well as in-frame deletions are also found in certain tumour types, resulting in constitutive activation of BRAF and downstream ERK signalling[12,13].

BRAF is commonly mutated in melanomas (50%)[14], papillary thyroid cancers (45%)[15], colon cancers (10%)[16], non-small-cell lung cancers (NSCLCs) (10%)[17], in virtually 100% of hairy cell leukemias[18] and in 50–60% of patients with the idiopathic disorder Langerhans cell histiocytosis (LCH)[19]. These findings, along with preclinical work demonstrating dependence of BRAF-V600E tumours on BRAF and ERK signalling activity[20], supported the therapeutic benefit of targeting ERK signalling in cancer and intensified efforts for the development of selective ATP-competitive inhibitors of mutant BRAF kinase. Two second-generation RAF inhibitors, vemurafenib and dabrafenib, showed remarkable clinical activity in patients with BRAF-V600E/K melanoma and received US Food and Drug Administration (FDA) approval for the treatment of this disease[21,22]. However, despite prolonging patient survival, RAF inhibitor treatment is rarely curative and is limited in most cases by the development of drug resistance and tumour relapse. As preclinical and clinical evidence suggested that more potent inhibition of ERK signalling in the tumour would yield improved responses[23], combinations of RAF inhibitors with MEK inhibitors were tested in subsequent trials. Both combinations (vemurafenib and cobimetinib (Roche/Genentech) and dabrafenib and trametinib (Novartis)) showed improved clinical efficacy compared with RAF inhibitor monotherapy[24,25]. Two combinations are now the standard of care for BRAF-V600E/K metastatic melanoma, although they are still not curative treatments for the majority of patients. Importantly, the most common mechanisms of resistance identified in tumours following relapse after combination RAF and MEK inhibitor therapy are associated with recovery of ERK signalling and are similar to the mechanisms of resistance detected after RAF inhibitor monotherapy[26-28]; this observation provides the rationale for the development of future therapeutic strategies that could achieve more potent inhibition of RAF–ERK signalling and may therefore yield deeper and more durable responses.

The remarkable clinical efficacy of currently approved RAF inhibitors has been so far confined to BRAF-V600E/K melanoma. Besides the most prevalent mutations in melanoma, BRAF-V600E (70–80%) and BRAF-V600K (5–12%), additional BRAF-V600 mutant forms have been identified in patients with melanoma but are less common. Among them, BRAF-V600R (3–7%) and the rare mutations BRAF-V600M, BRAF-V600D and BRAF-V600G (<5% in total) have been reported to be sensitive to approved RAF inhibitors in preclinical[29-32] and clinical[22,33-35] studies. By contrast, preclinical and clinical data suggested that colorectal and thyroid BRAF-V600E tumours, as well as tumours with BRAF mutations other than V600 substitutions, are less sensitive to current clinical RAF inhibitors[36-39].

In this Review, we highlight the most recent developments in our understanding of the biochemical basis of sensitivity and resistance to second-generation RAF inhibitors in cancer therapy. We will also discuss the ongoing development of third-generation RAF inhibitors with new structural properties designed to both address certain toxicities elicited by current clinical inhibitors and provide more durable therapeutic responses in patients with BRAF-mutant tumours.

## Structural insight into RAF activation

In normal cells and in the absence of upstream activity, cytosolic RAF is thought to adopt a monomeric, closed and inactive conformation due to the intramolecular interaction between the amino-terminal (regulatory) and the carboxy-terminal (kinase) domains[40,41] (FIG. 1a). Canonical RAF activation is regulated by members of the RAS family of small GTPases (KRAS, NRAS and HRAS), which, upon growth factor stimulation of upstream receptors, are converted from the inactive (RAS-GDP) to the active (RAS-GTP) form in the plasma membrane. The increase in the levels of RAS-GTP results in RAF translocation to the membrane and formation of the RAF–RAS-GTP complex, due to the high affinity of RAS-GTP for the RAS-binding domain (RBD) in the N terminus of RAF[42,43] (FIG. 1a). RAF kinases bound to RAS-GTP in the membrane become fully activated via priming (which includes phosphorylation at critical residues, such as S338 in CRAF) and homodimerization and heterodimerization through the kinase domain[44,45].

RAF has a typical kinase domain architecture, with the N-terminal lobe (N-lobe) and C-terminal lobe (C-lobe) linked through a short flexible hinge. At the interface of the two lobes lies the active site of the kinase that includes the nucleotide (ADP or ATP) binding site, the magnesium binding site (DFG motif), and the phospho-acceptor site (activation segment). The motions between the lobes are controlled by the flexible hinge and enable recruitment of the substrate and release of the product[46]. The catalytically active conformation of the kinase domain requires a closed conformation between the two lobes, an unfolded activation segment and the αC-helix of the N-terminal lobe fixed into the IN position to bring the catalytic residues into a productive distance and orientation (FIG. 1b). The active conformation in RAF is facilitated by dimerization. Dimerized RAF has been visualized by several crystal structures determined with BRAF or CRAF bound to inhibitors, providing insights into the dimerization interface[10,30,47-50]. The dimerization interface of each BRAF protomer includes the αC-helix of each kinase and specifically the interaction between the

C-terminal R509 residue of each αC-helix. Mutagenesis studies have established the critical role of R509 and adjacent residues in mediating BRAF dimerization[50-53].

Recently, two crystal structures of monomeric BRAF kinase bound to structurally related compounds[30,54] and the crystal structure of the BRAF–MEK complex[55] have been determined, thereby providing important insight on the conformational transitions and the structural basis of dimerization-dependent RAF activation. The structure of monomeric BRAF shows the kinase in an inactive conformation characterized by an open configuration between the N-lobe and C-lobe of the kinase domain[30,54]. The αC-helix is positioned in the OUT position, where it is stabilized by interactions with residues of the folded activation segment, which has also been determined[30,54] (FIG. 1c). The structure of dimeric BRAF kinase bound to MEK enabled the visualization of an active conformation of the BRAF structure and the closed configuration between the N-lobe and C-lobe of the kinase[55]. In this conformation, the position of the αC-helix is shifted to the full IN position, and the activation segment is extended as an unstructured loop that enables the shift of the αC-helix to facilitate catalysis and the interaction with the substrate[55]. The crystal structures show the DFG motif to adopt an IN conformation in both the active and the inactive kinase conformations[30,55] (FIG. 1b,c). These structural studies suggest that BRAF undergoes dynamic conformational transitions within the N-lobe, C-lobe, αC-helix and activation segment. These transitions are structurally interconnected and reveal how dimerization promotes activation of the kinase domain.

Despite a number of available crystal structures of either wild-type (WT) BRAF or BRAF-V600E, the detailed structural mechanism of BRAF activation by the most common BRAF mutation (V600 substitution) is still not well understood. Co-crystal structures of BRAF-V600E with RAF inhibitors suggested the potential for dimerization-induced activation by conformational changes promoted by the V600E substitution[10,54]. Recent studies suggested the importance of a salt bridge between V600E in the activation segment and K507 of the αC-helix in stabilizing the active conformation of BRAF-V600E as evidenced by crystal structures of the BRAF-V600E dimer[54,55]. However, cell-based and biochemical data established that BRAF-V600 proteins in the presence of low levels of RAS-GTP can be catalytically active monomers[30,31,51-53,56]. The reason for this discrepancy is presumably the absence of the N-terminal regulatory domain of BRAF in available crystal structures; this domain may be involved in the stabilization of an active monomeric conformation of V600-mutant BRAF kinase. Further studies are needed to elucidate the structural details of BRAF kinase activation. Structural understanding of the interaction of the N-terminal regulatory domain with the RAF kinase domain, in either the inactive conformation or the active conformation of the monomer, would be a major step towards elucidating the regulation of normal and oncogenic BRAF.

Beyond the physiological signalling mechanism of BRAF dimerization-induced activation through activated RAS, cell-based studies showed that certain oncogenic BRAF mutations promote spontaneous BRAF dimerization and activation, either by forming homodimers in the absence of RAS-GTP, such as BRAF-L597V or BRAF-G466E[11,30,31,51], or by further enhancing RAF dimerization promoted by RAS-GTP, such as the catalytically inactive BRAF-D594N mutant or ‘impaired activity’ mutants[57]. As in the case of BRAF-V600

mutants, the exact mechanism by which these mutations induce dimerization of the kinase is not well understood, due to the lack of structural information that includes the N-terminal domain of BRAF, or at least the part that regulates dimerization of the catalytic domain. Furthermore, several mutations are found in residues in the activation segment or in the glycine-rich loop, which interacts with the activation segment[9]. In most co-crystal structures of BRAF kinase, several residues of the activation segment are not resolved, precluding information of the position of these residues and their interactions in the activated BRAF structure. The recently reported structures of BRAF monomers in the inactive conformation suggest that mutation in the activation segment such as V600E may disrupt the hydrophobic packing between the inactive state of the activation segment and the αC-helix through steric clashes or unfavourable interactions; in turn, this can promote allosteric movement of the αC-helix towards the IN position, which eventually favours dimerization of the RAF kinase domain[30,54]. Additional crystal structures of BRAF mutants other than the V600 substitution are required for more definitive and detailed understanding of the mechanisms involved.

## Models of RAF inhibitor action

RAF inhibitors have unusual biochemical properties. Unlike most kinase inhibitors, which suppress their targets in all cells, current clinical RAF inhibitors suppress RAF activity and ERK signalling selectively in cells expressing mutant BRAF. In tumour and normal cells expressing WT BRAF, these RAF inhibitors do not inhibit but instead paradoxically activate RAF activity and downstream ERK signalling (the so-called ‘RAF inhibitor paradox’)[1,47,49,56-63]. The complex biochemical mechanism of action of RAF inhibitors has been the topic of intense investigation, and it was only recently, with the integration of structural, biochemical and cell-based analysis of a large number of structurally diverse RAF inhibitors, that it has been understood in more detail.

### Structural insight into RAF inhibitor activity

Dependent on their chemical structure, RAF inhibitors stabilize the αC-helix in a position between the IN and OUT conformations, forming imperfect dimers due to differences in the position of the αC-helix within each protomer (FIG. 2a). A notable example is vemurafenib, which binds the first protomer and stabilizes the αC- helix towards the OUT position. In the second protomer, the IN position of the αC-helix creates negative allostery for binding a second vemurafenib molecule, because movement of the αC-helix towards the OUT position in that protomer would cause disruption of the dimer (Supplementary information S1 (movie)). By contrast, other RAF inhibitors, such as AZ628, that stabilize the αC-helix of the first protomer towards the IN position, sterically allow occupancy of the second protomer by a second drug molecule (Supplementary information S2 (movie)). Thus, RAF kinase inhibitors can be broadly classified according to the conformation in which they stabilize their target kinase — ‘αC-IN’ (either αC-helix-IN/DFG-IN (type I) or αC-helix-IN/DFG-OUT (type II)) or ‘αC-OUT’, the latter most commonly occurring as αC-helix-OUT/DFG-IN (type $I^{1/2}$)[64], which is the class to which the current clinical RAF inhibitors vemurafenib and dabrafenib belong (FIG. 2a and TABLE 1).

## Linking structure to biochemical effects of RAF inhibitors in cells

The first studies focusing on the mechanisms underlying the unique biochemical properties of RAF inhibitors were published during the preclinical and clinical development of inhibitors targeting mutated BRAF[47,56,57]. Although not all studies concurred in their proposed models, they all agreed that paradoxical activation of ERK signalling by RAF inhibitors in WT BRAF-expressing cells required active RAS. However, the details of this mechanism remained elusive. Furthermore, in those and subsequent studies, RAF dimerization was shown to confer resistance to RAF inhibitors[31,52], but the structural basis of the phenomenon remained unknown. More recently, detailed analysis of crystallographic data in combination with cell-based studies enabled the structural properties of RAF inhibitors to be linked to their biochemical effects in cells[30,49] (FIG. 2b,c). Specifically, the biochemical effect of RAF inhibitors is the outcome of distinct allosteric mechanisms, determined by the combination of the specific conformation in which the RAF kinase is stabilized by the inhibitor and by the cellular levels of RAS-GTP.

First, resistance of dimeric RAF to inhibitors is the consequence of stabilization of the αC-helix in the OUT (inactive) position by the inhibitor, because this position of the αC-helix is not sterically allowed for both protomers in a RAF dimer. Thus, binding of an αC-OUT inhibitor to the first protomer stabilizes the position of the αC-helix of the second protomer towards the IN position, reducing the affinity of a second αC-OUT inhibitor for the second protomer (negative allostery). Such inhibitors are predicted to be ineffective inhibitors of dimeric RAF[30] (FIG. 2b). Certain αC-OUT RAF inhibitors, such as dabrafenib, stabilize the αC-helix in a less OUT position compared to vemurafenib in both protomers, a position that allows the inhibitor to bind both protomers, as seen in the crystal structure with BRAF kinase. However, this might be an effect of crystallization forcing the two protomers into a ‘distorted’ dimer. In contrast, αC-IN RAF inhibitors bind both protomers of dimeric RAF with similar affinity and are thus predicted to be equipotent inhibitors of monomeric and dimeric RAF[30] (FIG. 2c). Because CRAF signals as an obligatory dimer[50,65], αC-OUT inhibitors fail to suppress its activity. Thus, αC-IN RAF inhibitors, by suppressing both BRAF and CRAF dimers can also be considered ‘pan-RAF inhibitors’[66-68] and may be effective when used in combination in contexts such as RAS-mutant tumour cells, in which CRAF is known to be a major activator of ERK signalling downstream of RAS. However, a potentially narrow therapeutic window and allosteric priming (see below) are predicted to limit the effectiveness of αC-IN RAF inhibitor monotherapy in RAS-mutant tumours[30]. Although the model of negative allostery due to the OUT position of the αC-helix is mostly inferred based on structural and cell-based data and remains to be shown directly by biophysical methods, it helps explain why αC-OUT inhibitors are selectively effective in cellular contexts in which RAF signals as a monomer (that is, BRAF-V600 tumour cells with low RAS-GTP) and why increased RAF dimerization is a common mechanism of resistance to these drugs[30].

Second, the basis for priming and RAS-dependent dimerization of RAF promoted by RAF inhibitors is the stabilization of the αC-helix towards the IN (active) position. αC-IN RAF inhibitor binding to RAF promotes the interaction of RAF with RAS-GTP, increasing activating phosphorylation and RAF dimerization, and essentially ‘hijacking’ the process of

canonical growth factor-induced RAF activation[30]. The mechanism is reminiscent of allosteric priming by inhibitor, which has been observed with other kinase inhibitors (such as those targeting AKT[69], protein kinase C (PKC)[70], ribosomal 6-kinase 1 (S6K1)[71] and Janus kinase 2 (JAK2)[72]), although in these cases virtually all the primed and phosphorylated kinase molecules are drug-bound and inhibited. Analysis of RAF priming by various inhibitors, led to the hypothesis that it is the area around a specific amino acid of the αC-helix (R506) and not the overall position of the αC-helix that determines the extent of the RAF–RAS-GTP interaction induced by the inhibitor[30]. According to this model, αC-IN inhibitors, such as GDC0879 or SB590885, displace R506 from the OUT position and thus promote RAF–RAS-GTP interaction[30]. Importantly, dabrafenib and to a lesser extent vemurafenib, although they stabilize the αC-helix in an overall OUT position, also promote RAF–RAS-GTP interaction and RAF dimerization due to displacement of the R506 residue towards the IN position[30]. Members of a certain class of next-generation inhibitors (such as PLX7904 and PLX8394; see below for more details) stabilize the entire αC-helix, including the R506 residue, in the OUT position and thus do not promote paradoxical activation of ERK signalling in cells[30,73]. Although further experimental data are required to substantiate this hypothesis, it may explain the requirement of RAS-GTP for paradoxical activation of ERK signalling by inhibitors and the concurrent finding of mutant-RAS expression in the squamous-cell carcinomas and keratoacanthomas, commonly induced as second-site tumours in patients with melanoma treated with second-generation RAF inhibitors[74].

Based on this model, paradoxical activation of ERK signalling by RAF inhibitors is the consequence of inhibitor binding to RAF, which in turn promotes RAF binding to RAS-GTP and results in an increase in active RAF dimers. Overall, RAF dimers are ineffectively inhibited by RAF inhibitors due to negative allostery for binding to the second protomer (FIG. 2b). αC-IN RAF inhibitors promote the RAS–RAF interaction at lower concentrations and effectively suppress ERK signalling at moderate and high concentrations, due to lower negative allostery (FIG. 2c). In contrast, αC-OUT inhibitors promote the RAS–RAF interaction at moderate and high concentrations and fail to suppress ERK signalling at these concentrations due to higher negative allostery[30].

Finally, there are data indicating that the position of the αC-helix and the DFG motif affect the interaction of RAF with MEK. αC-OUT RAF inhibitors (for example, vemurafenib or dabrafenib) tend to disrupt the RAF–MEK complex. Among αC-IN RAF inhibitors, those stabilizing a conformation closer to the native active kinase (αC-IN/DFG-IN, for example, GDC0879 or SB590885) allosterically promote the formation of the RAF–MEK complex and thus display more pronounced paradoxical ERK signalling activation than do αC-IN/DFG-OUT inhibitors[30,55].

It should be emphasized that despite the major contributions by a number of groups to our understanding of the complex mechanism of action of RAF inhibitors, more work is required to further elucidate the highly dynamic structural rearrangement that RAF proteins undergo upon inhibitor binding. Adding to this complexity is the variation in the details of the mechanism of action, even among inhibitors of the same class, and for example, as a result of the mutational status of the RAF proteins, the cell-specific stoichiometry of the various RAF interacting proteins and the levels of RAS activity.

Nonetheless, the mechanism of action of RAF inhibitors has important clinical implications. First, αC-OUT RAF inhibitors are predicted to be effective selectively against monomeric forms of RAF, but any mechanism of RAF dimerization will cause drug resistance, a notion that has been confirmed both in the laboratory and in the clinic[30,31,62,75]. Second, αC-IN RAF inhibitors, by inhibiting both monomeric and dimeric RAF to a similar extent, will overcome resistance due to RAF dimerization. However, they are predicted to have a fairly narrow therapeutic window, given that they will suppress ERK signalling in normal, WT BRAF-expressing cells at concentrations similar to those used to suppress ERK signalling in tumour cells, as suggested by preclinical data[30].

## Activation of mutant BRAF

### Linking BRAF mutational alterations to predicted RAF inhibitor response

A critical breakthrough in elucidating the mechanism of action of RAF inhibitors was the discovery that, unlike WT BRAF that requires dimerization for its catalytic activation, mutant BRAF-V600E is able to signal as an active monomer in the absence of RAS-GTP[30,31,51,52,56]. These studies were carried out using ectopically expressed proteins and mutational analysis; thus, the cellular status and the composition of endogenous BRAF-V600E containing complexes have yet to be unequivocally determined[53,76]. Nonetheless, the basis for the wide clinical therapeutic window of the current clinical αC-OUT RAF inhibitors vemurafenib and dabrafenib is believed to be their relative inability to inhibit dimeric RAF in normal cells, while potently inhibiting mutant BRAF in tumour cells.

Recent work has revealed important differences in the mechanism by which BRAF mutations other than BRAF-V600E promote catalytic activation of BRAF (FIG. 3). First, similar to BRAF-V600E, other BRAF-V600 point mutant forms, such as V600K, V600D and V600R, are also catalytically active monomers in the absence of RAS-GTP and are sensitive to inhibition by current clinical αC-OUT RAF inhibitors[30,31]. By contrast, the catalytic activity of non-V600 BRAF point mutants depends either partially or fully on dimerization[30,31,51]. Thus, non-V600 BRAF mutants, including BRAF-L597V, BRAF-K601E and BRAF-G466E, signal as RAS-GTP-independent RAF dimers by spontaneously dimerizing, and they are thus resistant to vemurafenib and other αC-OUT inhibitors[11,30,31,51]. However, this has been shown only in cell-based experiments so far[30,31,51]. The structural basis for the difference between RAF inhibitor activity upon V600 and non-V600 BRAF point mutants will presumably require biochemical and crystallographic studies including the N-terminal domain of BRAF or at least the part of the N-terminal domain that regulates RAF dimerization. Nonetheless, as with other forms of dimeric RAF, αC-OUT RAF inhibitors are not predicted to be effective in tumours with non-V600 mutant BRAF. Equipotent inhibition of monomeric and dimeric RAF with αC-IN RAF inhibitors may be a promising alternative for this class of tumours[30,31].

In addition to point mutations, BRAF (and more rarely CRAF) is found as part of fusion transcripts and proteins caused by translocations[77]. Such fusions are commonly found in paediatric gliomas[78] but also in other solid tumours, including lung cancers, advanced prostate cancers, gastric cancers, pancreatic cancers and melanoma[77,79]. Typically, translation of the fusion transcript results in the expression of RAF proteins either as the

intact kinase domain only or as the kinase domain fused with a partner protein that has replaced the N-terminal domain of RAF[77]. Deletion of the N-terminal domain of RAF has been shown to promote RAF activation by constitutive dimerization[44,52]; thus, RAF fusion proteins are predicted to be resistant to αC-OUT RAF inhibitors. As αC-IN RAF inhibitors potently suppress dimeric RAF, they may be a therapeutic option in this context.

In-frame deletions in BRAF, such as L485–P490-deleted BRAF and N486–P490-deleted BRAF, have also been found in a small percentage of certain tumours, with the highest prevalence in pancreatic carcinomas (4.21%), as well as in lung, ovarian and thyroid tumours[80]. BRAF in-frame deletions do not overlap with KRAS or other BRAF mutations[80], suggesting that they are sufficient to drive ERK signalling hyperactivation in these tumours. The deletions are mapped within the conserved β3/αC-helix loop of the kinase domain of BRAF, and they are predicted to activate the kinase by shortening the β3/αC-helix loop in a fashion analogous to that of in-frame deletions in epidermal growth factor receptor (EGFR) and HER2 (also known as ERBB2), also found in tumours[12]. This shortening of the β3/αC-helix loop results in a unique conformation that locks the αC-helix in the IN position through the formation of an intact K483-to-E501 salt bridge. Two recent studies focused on the mechanism of activation of in-frame BRAF deletions and their response to RAF inhibitors[12,80]. Chen *et al.*[80] proposed that the increased activity of these genetic alterations is caused by increased BRAF homodimerization. Therefore, BRAF proteins carrying in-frame deletions are maintained in the active state that favours BRAF homodimerization and were shown to be resistant to vemurafenib but sensitive to the next-generation αC-IN RAF dimer inhibitor LY3009120 (REF. 80) (see below for more detail). By contrast, Foster *et al.*[12] suggested that the activity of BRAF deletions is independent of RAF dimerization and that these in-frame-deletion mutants can signal as monomers, similar to BRAF-V600E. Structural analysis revealed that the αC-helix-IN active conformation of the kinase domain of BRAF harbouring a deletion, renders binding of vemurafenib incompatible due to steric effects[12]. Although the two studies differ on the proposed mechanism of BRAF activation by in-frame deletions, they both conclude that tumours expressing BRAF deletions will be resistant to current clinical RAF inhibitors and likely to respond to equipotent inhibitors of monomeric and dimeric RAF[12,80].

Finally, there are a class of point mutations found in either the activation segment or the glycine-rich loop of BRAF that are not activating but that instead result in a protein that either is catalytically inactive or shows severely impaired activity compared to WT BRAF[10,11,57]. These forms of mutant BRAF with impaired activity have been found mainly in colorectal, melanoma and lung tumours[81], require RAS activity and dimerization to hyperactivate ERK signalling and transform cells[11,31,57] and are commonly co-expressed with activated receptor tyrosine kinases (RTKs) or upstream alterations of RAF signalling, such as RAS mutations or neurofibromin 1 (NF1) loss[82,83]. As these mutant BRAF proteins form dimers, tumours harbouring them are predicted to be insensitive to current clinical αC-OUT RAF inhibitors, but αC-IN RAF inhibitors may be an effective option.

### Mechanisms of resistance to RAF inhibitors

The notion that second-generation RAF inhibitors do not effectively suppress dimeric RAF was emphatically confirmed with the discovery of increased RAF dimerization as a common cause of clinical acquired resistance to RAF inhibitors[30,31,52,75,84]. Various molecular mechanisms have been identified that confer this clinical resistance to second-generation RAF inhibitors. RAS activation[75], either by RAS mutation or by overexpression of upstream RTKs[75,85], promotes RAF dimerization as well as activation of parallel survival pathways, such as PI3K–AKT signalling. BRAF-V600E amplification has been found in a subset of resistant melanoma tumours[28,84], with the resulting overexpression of the BRAF-V600E protein predicted to promote resistance to RAF inhibitors by both increasing abundance of the target (that is, BRAF-V600E) and spontaneous BRAF-V600E dimerization[31]. Another common mechanism of clinical resistance due to increased BRAF-V600E dimerization is the expression of splice variants of BRAF-V600E that lack exons encoding part of the N terminus of the protein, specifically those including the RBD, which prevents dimerization in the context of full-length BRAF[52,86]. Finally, mutational activation of MEK has also been reported in a subset of BRAF-V600E tumours that developed resistance to RAF inhibitors[87,88].

In addition to acquired resistance, increased RAF dimerization may confer an adaptive response to RAF inhibitor therapy. In BRAF-V600E-expressing tumour cells, any increase in RAS activity due to upstream activation will cause formation of RAF dimers, including homodimers and heterodimers of WT and BRAF-V600E, CRAF and BRAF-V600E and ARAF and BRAF-V600E, thus hindering the effect of RAF inhibitors[30,89]. A number of studies have proposed mechanisms by which tumours may adapt to RAF inhibitor therapy via upstream RTK and RAS activation, either as a result of relief of negative feedback[37-39,89] or due to cytokine and growth factor release from the tumour microenvironment[90-92] (FIG. 4).

In tumour types other than melanomas harbouring BRAF-V600E mutations, such as in colorectal and thyroid cancers, the response rates to second-generation RAF inhibitors are low[93,94]. In these tumours, increased upstream RTK expression and activity at baseline compared to melanoma (mostly through EGFR in colorectal cancers and HER2–HER3 (also known as ERBB3) signalling in thyroid cancers) has been reported to account for the unexpected unresponsiveness[37-39]. Consequently, feedback reactivation of upstream RTKs and RAS upon RAF inhibitor treatment is more pronounced in these tumours, profoundly enhancing RAF dimerization and thus limiting the effect of αC-OUT inhibitors[30].

## Development of RAF inhibitors

### First-generation (αC-IN) RAF inhibitors

The first small-molecule ATP-competitive RAF inhibitors were reported before the discovery of BRAF mutations and were developed with the goal of targeting CRAF in cancer[95]. The compound ZM336372 (likely to be αC-IN/DFG-IN) was identified from a chemical screen as both a CRAF and BRAF inhibitor *in vitro* and was associated with the first report of the phenomenon of paradoxical ERK activation induced by a RAF inhibitor in

cells[95]. Sorafenib (Nexavar, BAY43-9006; Bayer/Onyx Pharmaceuticals) (αC-IN/DFG-OUT) is the only first-generation RAF inhibitor that advanced to the clinic[96] (and received FDA approval for the treatment of advanced renal cell carcinoma and hepatocellular carcinoma[97,98]). Sorafenib shows weak activity against BRAF-V600E in cells, and its clinical activity in the aforementioned tumours with WT BRAF is likely due to its multi-kinase profile. Other first-generation RAF inhibitors that did not advance to the clinic include SB590885 (REF. 99) and GDC0879 (REF. 100) (both αC-IN/DFG-IN) and GW5074 (REFS 101,102) and L779450 (REF. 87) (there are currently no crystal structures for GW5074 and L779450, but it is likely they are αC-IN/DFG-IN).

### Second-generation, BRAF-V600E-selective (αC-OUT) RAF inhibitors

The discovery of BRAF mutations in 2002 (REF. 9) renewed interest in developing RAF inhibitors, leading to second-generation compounds, which were identified after screening for inhibitors of BRAF-V600E[48]. The first such RAF inhibitor to enter clinical trials was vemurafenib (Zelboraf, PLX4032; Plexxikon/Genentech), developed by a structure-guided discovery approach[23,48]. Vemurafenib was approved by the FDA in 2011 for the treatment of patients with BRAF-V600E metastatic melanoma[22,33], followed by dabrafenib (Tafinlar, GSK2118436; GlaxoSmithKline), the second RAF inhibitor to receive approval by the FDA in 2013 for the treatment of melanoma patients with BRAF-V600E/K mutations[21,103]. The two inhibitors showed similar clinical efficacy and induced similar on-target effects related to paradoxical activation of ERK signalling in normal cells. Approximately 14–26% of patients with melanoma treated with second-generation RAF inhibitors were diagnosed with the secondary cancers keratoacanthomas and squamous-cell carcinomas, which can be treated by simple excision[74]. These second- site cancers are commonly associated with expression of a RAS mutation and have been almost eliminated with the combination of a RAF and a MEK inhibi tor, which is the current standard of care (see below). Other side effects, such as photosensitivity caused by vemurafenib[22] and fever caused by dabrafenib[21], appear to be more drug-specific, presumably related to different off-target effects of the two drugs.

LGX818 (Encorafenib, Array BioPharma) is another RAF inhibitor selective for mutant BRAF that has shown promising clinical activity and is currently in phase III clinical trials[104]. Preclinically, the cellular profile of the compound is similar to other αC-OUT inhibitors. It has been reported to show longer residence time (lower offrate) compared to vemurafenib or dabrafenib[31], a property that may positively affect its clinical profile by prolonging target inhibition.

### Third-generation RAF inhibitors

Third-generation RAF inhibitors, which are currently under preclinical and clinical development, fall structurally and biochemically into two main classes (TABLE 1). One group includes compounds that equipotently inhibit the monomeric and dimeric forms of RAF and are thus expected to overcome resistance due to RAF dimerization. Among them are TAK632 (REF. 105) and MLN2480 (TAK580)[106] (Takeda/Millennium), LY3009120 (REF. 68) (Eli Lilly) and CCT3833 (BAL3833) (REF. 107) (ICR/Royal Marsden NHS Foundation Trust). Crystallographic data obtained with TAK632 or LY3009120 bound to

BRAF show a type II conformation (αC-IN/DFG-OUT), which explains the similar affinity for both protomers in the RAF dimer, as well as the generally slow off-rate of these compounds[67,68,105].

In preclinical studies, TAK632 demonstrated potent antiproliferative activities in multiple cancer cell lines harbouring *BRAF, NRAS* or *KRAS* mutations with the potential to delay the emergence of resistance[30,67]. The co-crystal structure of TAK632 bound to WT BRAF revealed compound occupancy and similar conformations of both protomers of the BRAF dimer[105]. TAK632 exhibited equipotent inhibition of monomeric and dimeric RAF in cells[30,67] and is expected to be effective in vemurafenib-resistant melanomas harbouring BRAF or NRAS mutations and BRAF-V600E colorectal or thyroid tumours[67]. MLN2480, which has characteristics similar to TAK632, is currently in a phase I clinical trial for patients with melanomas and other solid tumours[108]. MLN2480 will also be evaluated in a phase I clinical trial in combination with the programmed cell death 1 (PD1) monoclonal antibody (nivolumab) for patients with melanoma[109]. Finally, a phase I clinical trial of MLN2480 in combination with inhibitors targeting other families of kinases (MLN0128, an mTOR complex 1 (mTORC1) and mTORC2 inhibitor, and alisertib, an Aurora kinase A inhibitor), chemotherapy regimens (paclitaxel and irinotecan) or the EGFR monoclonal antibody (cetuximab) will be conducted for patients with advanced solid malignancies[110].

LY3009120 is another αC-IN/DFG-OUT RAF inhibitor that showed antitumour activity in preclinical studies against NRAS or KRAS mutant tumours and vemurafenib-resistant melanomas[68,111]. LY3009120 binds to both RAF protomers within the BRAF dimer, stabilizing them in similar conformations, and, like other αC-IN RAF inhibitors, it inhibits monomeric and dimeric BRAF with similar potency[30,41,93]. LY3009120 was also effective against BRAF deletions, including BRAF-N486–P490 and other in-frame BRAF deletions that have been identified in pancreatic and thyroid tumours[80]. Currently, LY3009120 is in a phase I clinical trial for patients with advanced cancers[112].

A compound from BeiGene, BGB659, showed properties of equipotent inhibitors of monomeric and dimeric RAF[31]. In preclinical studies, BGB659 was effective in melanomas with V600E and non-V600 BRAF mutations, in vemurafenib-resistant BRAF-V600E melanomas, as well as against ectopically expressed BRAF fusions[31]. A related compound, BGB283, acts as a dual RAF and EGFR inhibitor[113]. In preclinical studies, it suppressed the proliferation of cells harbouring BRAF-V600E or EGFR mutations and inhibited EGFR reactivation in BRAF-V600E colorectal tumours. A phase I clinical trial of BGB283 for patients with solid tumours is ongoing[114].

CCT196969 and CCT241161 have been reported as dual pan-RAF and SRC kinase inhibitors[66]. Although crystallographic data are not available, these compounds behave biochemically in a similar manner to αC-IN RAF inhibitors. In preclinical studies, both CCT196969 and CCT241161 inhibited the growth of BRAF-V600E melanomas as well as RAS-mutant melanomas and colorectal tumours[66]. CCT196969 and CCT241161 were also effective in patient-derived xenograft (PDX) models that included melanomas with intrinsic or acquired resistance to second-generation RAF and MEK inhibitors[66]. The study of the safety and tolerability of the lead compound of this class of inhibitors, CCT3833

(BAL3833), is ongoing in a phase I clinical trial for patients with advanced solid tumours[107]. Melanoma patients harbouring BRAF-V600E (either treatment-naive or after progression on RAF inhibitor therapy) or RAS mutations are also included in the trial. Finally, LXH254 and RAF709, both currently under development by Novartis are also equipotent inhibitors of monomeric and dimeric RAF. An ongoing phase I clinical trial is recruiting patients with tumours with ERK signalling alterations to be treated with LXH254 (REF. 115).

The other group of third-generation RAF inhibitors includes the ‘paradox breakers’, which are αC-OUT RAF-inhibitor derivatives with variable terminal sulfonamide and sulfamide substitutions. Both PLX7904 and its analogue PLX8394 (Plexxikon) are more potent inhibitors of BRAF-V600E than vemurafenib, and unlike vemurafenib, they do not promote paradoxical ERK activation in WT BRAF cells or squamous-cell carcinomas in preclinical models[73]. These compounds stabilize the αC-helix of BRAF in the OUT position, and structural analysis showed that the position of the R506 residue of the αC-helix with PLX7904 has the furthest OUT position compared to that with non-paradox breakers[30,73]. Thus, the ‘paradox breakers’ are predicted to have fewer on-target toxicities than second-generation RAF inhibitors and may be an effective option for chronic administration in more indolent neoplasias, such as in LCH patients with tumours expressing BRAF-V600E[19]. PLX8394 is in a phase I/II clinical trial for the treatment of patients with advanced unresectable solid tumours[116]. Preclinical studies using ‘paradox breakers’ have also suggested that they will be effective against vemurafenib-resistant tumours and BRAF fusions that are common in paediatric astrocytomas, presumably due to their higher potency against RAF when compared with vemurafenib[117-119]. However, as they are αC-OUT inhibitors, RAF dimerization is predicted to limit the effectiveness of these compounds.

Finally, BI882370 is an αC-OUT RAF inhibitor under development by Boehringer Ingelheim that showed promising preclinical activity against BRAF-mutant melanoma and colorectal tumours, both in cell line models and *in vivo*[120]. This compound differs from other αC-OUT RAF inhibitors by stabilizing a conformation in which it interacts with the Phe595 from the DFG motif and keeps the DFG motif in an inactive (OUT) position[120]. However, further studies are required to determine potential functional consequences of these differences.

Currently in clinical trials are additional RAF inhibitors that cannot yet be classified due to a lack of structural data, including HM95573 (Hanmi Pharmaceutical/Genentech) and CEP32496 (RXDX-105, Ambit Biosciences/Ignyta). HM95573 is currently in an expansion clinical trial for solid tumours harbouring mutations in either *BRAF, KRAS* or *NRAS* genes[121,122]. CEP32496 (REF. 123) is a quinazoline BRAF inhibitor that has demonstrated multikinase activity against BRAF-V600E, BCR-ABL, RET and ephrin type A receptor 2 (EPHA2) and is reported to be effective in preclinical studies of melanomas and colorectal tumours harbouring BRAF-V600E[123]. CEP32496 will be evaluated in a phase I clinical trial for the treatment of patients with solid tumours[124].

Based on preclinical data, clinical efficacy of next-generation RAF inhibitors may also be limited by either on-target toxicities or development of drug resistance. Third-generation αC-OUT RAF inhibitors (such as the ‘paradox breakers’) are expected to retain the wide

therapeutic window of second-generation αC-OUT RAF inhibitors, without the on-target toxicities of second-site tumours due to paradoxical ERK activation in normal cells. However, RAF dimerization is predicted to limit the effectiveness of these inhibitors. In contrast, αC-IN RAF inhibitors are predicted to overcome resistance due to RAF dimerization, but resistance to these compounds is anticipated to be conferred by other mechanisms, such as other mutations in BRAF or alterations that would enable cell proliferation and survival in the absence of ERK signalling. However, allosteric priming and a fairly narrow therapeutic window due to inhibition of ERK signalling in normal cells at lower drug concentrations is expected to limit the effectiveness of αC-IN RAF inhibitors. Therapeutic strategies combining structurally complementary RAF inhibitors with inhibitors of downstream components of ERK signalling (MEK and ERK inhibitors) may enable sufficient inhibition of ERK signalling in the tumour and thus provide durable responses and perhaps even cures for patients with BRAF-mutant tumours.

## Combinatorial therapeutic strategies

As in both preclinical models and in patients, potency of ERK inhibition in the tumour was found to correlate with response to RAF inhibitor treatment[23], RAF inhibitors were combined with MEK inhibitors in melanoma patients with BRAF-V600E tumours in order to achieve more potent and durable inhibition of ERK signalling in the tumour[24,25]. RAF–MEK inhibitor combinations demonstrated increased progression-free survival (PFS) and objective responses compared to RAF inhibitor monotherapy[24,25]. Moreover, the cutaneous toxicities induced by RAF inhibitors were dramatically decreased in the combination, further confirming that they were the result of paradoxical activation of ERK signalling in normal tissue. The dabrafenib and trametinib combination[24] (Novartis) increased the response rate to 76% and the median duration of response to 10.5 months as compared with a 54% response rate and 5.8 months duration of response with dabrafenib alone, and was approved by the FDA in 2014. The combination of vemurafenib and cobimetinib[25] (Roche/Genentech) demonstrated similar responses (response rate 68% and duration of response 9.9 months as compared with response rate of 45% and median duration of response of 6.2 months in patients treated with vemurafenib alone) and was also approved by the FDA in 2015. Given their superior efficacy over RAF inhibitor monotherapies, these RAF–MEK inhibitor combinations are currently the standard of practice for the treatment of melanoma patients with BRAF-V600E/K mutations.

Clinical trials of drug combinations including the second-generation RAF inhibitor LGX818 are currently underway: one with the MEK inhibitor MEK162 (binimetinib, Array BioPharma) in patients with BRAF-V600E melanomas (COLUMBUS)[104], and another in combination with MEK162 and the EGFR antibody cetuximab in patients with colorectal BRAF-V600E tumours (BEACON CRC)[125]. Preliminary results from the COLUMBUS trial presented on the Array BioPharma website indicate significant responses, including increased median PFS and improvement in overall response rate compared with vemurafenib monotherapy.

Despite the improvement of clinical outcome, responses to RAF–MEK inhibitor combinations are variable, and resistance develops for the majority of patients treated with

these regimens. Most mechanisms of resistance to RAF–MEK inhibitor combination are similar to the ones identified with RAF inhibitor monotherapy and are associated with recovery of ERK signalling in the presence of inhibitors[27,126]. This suggests that strategies that would more potently and durably suppress RAF–ERK signalling in the tumour, while retaining a wide therapeutic window, may yield even better responses and further forestall drug resistance. The wide therapeutic window of αC-OUT RAF inhibitors suggests that they will most likely remain components of any future combinatorial strategy for BRAF-V600E tumours.

A number of next-generation αC-IN RAF inhibitors are currently in clinical trials (TABLE 1). Such compounds are predicted to suppress dimeric RAF and thus be more effective in a broad range of RAF-dependent tumours, including tumours expressing non-V600 BRAF mutations, or perhaps RAS-mutant tumours. However, the narrow therapeutic window of these inhibitors may be a critical factor limiting their efficacy as single agents. As aforementioned, adding a modest dose of an αC-IN RAF inhibitor to the current standard αC-OUT–MEK inhibitor combination may be an effective strategy for patients with BRAF-V600E tumours[30]. Preclinical work supports the idea of including αC-IN RAF inhibitors in combination with αC-OUT RAF inhibitors (and also MEK or ERK inhibitors) to overcome feedback recovery and thus achieve durable suppression of ERK signalling[30,127].

RAF inhibitors are also included in a number of trials that are undertaking a combinatorial approach to target parallel pathways (for example, RTKs, cyclin-dependent kinase 4 (CDK4) and CDK6 and PI3K) in addition to ERK signalling[128-131]. Although in principle, these strategies are usually based on strong preclinical rationale, additive on-target and off-target toxicities tend to result in lower dosing and thus limit the efficacy of the individual compounds in the clinic. Again, it is critical that the need for a wide therapeutic window be taken into account in the design of clinical trials assessing combinations of targeted agents.

Finally, a great deal of interest is being directed towards elucidating the combinatorial interaction of targeted therapy with immunotherapy, especially in melanoma treatment. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and PD1 inhibitors have shown remarkable clinical efficacy and prolonged survival of patients with melanoma[132-134] and are now FDA-approved for the treatment of metastatic melanoma[135]. Trials with concomitant administration of immune checkpoint inhibitors and RAF and MEK inhibitors have so far been discontinued due to unacceptable adverse effects, although it is not inconceivable that certain combinations and scheduling may be better tolerated than others. If simultaneous treatment is not an option, whether immunotherapy or targeted therapy should be the first-line treatment for patients with BRAF-V600E melanomas remains an open question, as there is reported evidence for cross-resistance[136]. In current clinical practice, targeted therapy is the preferred first-line therapy for rapidly growing tumours because it tends to yield faster remissions than immunotherapy, but a number of ongoing clinical trials are aimed at exploring this question more broadly[137].

## Challenges and future perspectives

Since the validation of BRAF-V600E as a drug target, as well as outcomes from sequencing projects highlighting the role of *BRAF*$^{V600E}$ and other *BRAF* mutations in several cancers, drug discovery and development of BRAF inhibitors has progressed at a fast pace. However, there is a pressing need for more effective RAF signalling inhibition that would overcome resistance due to mechanisms of BRAF dimerization. As our understanding of the structural and biochemical regulation of BRAF progresses, novel and more specific targeting strategies should be developed. Future efforts should explore the possibility for improving current ATP-competitive αC-OUT inhibitors to prevent negative allostery and bypass paradoxical activation. In addition, third-generation αC-IN inhibitors are more effective inhibitors of RAF dimers than are αC-OUT inhibitors, although their selectivity for mutant or WT BRAF dimers versus BRAF monomers is not optimal. Finally, one could envision the development of allosteric inhibitors that effectively inhibit the mutant or WT BRAF dimer and/or BRAF monomer but that do not promote the RAF priming that leads to paradoxical activation.

Key biochemical data suggest the importance of the BRAF–CRAF heterodimer in RAF oncogenic signalling[56,57,65,138], although the structure of this heterodimer has not yet been determined. Nevertheless, peptides generated from the dimerization interface of BRAF have been shown to prevent formation of homodimers and heterodimers of BRAF–CRAF in a BRAF-mutant or RAS-mutant cellular context[51], suggesting the possibility of an alternative strategy for developing inhibitors of RAF dimerization. Lastly, we mentioned that several BRAF mutations other than V600 can spontaneously induce BRAF dimerization through the kinase domain[30,31,51]. Structures of such BRAF mutants favouring dimer formation are not available and should provide further understanding about their mechanism of activation, as well as serve as templates for tailored drug design to these mutations.

Importantly, the structural basis of the interaction of the N-terminal regulatory domain and the kinase domain in the inactive conformation and the active conformation remains elusive. Such information would enable our understanding of the architectural organization of BRAF and the conformational transitions involved in the context of the full-length BRAF from its inactive monomer conformation to the active dimer and active monomer, particularly in the case of the BRAF-V600 mutant. The effects of the current inhibitors upon these inter-domain interactions in the active and inactive BRAF conformations are not well understood. Given the conformational changes in the kinase domain and dimerization process observed with the current BRAF inhibitors, it is likely that these same inhibitors will have an impact on inter-domain interactions. Such information will enable further improvement of the current inhibitors but will also provide unique opportunities for BRAF allosteric inhibitors outside of the catalytic pocket.

The oncoprotein BRAF is a validated therapeutic target in a large number of human tumours. Current clinical RAF inhibitors have improved survival of patients with melanoma whose tumours harbour *BRAF* mutations, but resistance limits their effectiveness in these patients, as well as in patients with BRAF-V600 tumours other than melanomas, or harbouring *BRAF* mutations other than V600. Our deeper understanding of the molecular and biochemical mechanisms of resistance to current clinical RAF inhibitors, combined with

the development of next-generation RAF inhibitors with different structural and biochemical properties, will enable the design of innovative, potentially more effective, RAF-inhibitor-based therapeutic strategies tailored to the clinical context.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Glossary

**Langerhans cell histiocytosis (LCH)**
A myeloid neoplasia characterized by inflammatory lesions containing pathological dendritic cells, frequently harbouring the BRAF-V600E mutation.

**Protomer**
The structural unit of an oligomeric protein; in the case of wild-type BRAF, a functional BRAF dimer is composed of two protomers.

**Steric clashes**
When atoms from different residues come into close proximity, the resultant repulsion between the atoms leads to a change in conformation.

**Negative allostery**
Also known as allosteric inhibition, occurs when binding of one ligand to a substrate (in this case a BRAF protomer) decreases the affinity of another ligand at a different site (that is, the other protomer).

**Therapeutic window**
The range of concentrations of a drug in the patient that provides safe effective therapy. The therapeutic window is wide when the minimum toxic concentration is much higher than the minimum effective concentration.

**Allosteric priming**
A phenomenon recently observed with several small-molecule inhibitors, by which binding of the inhibitor induces the active conformation of the target kinase, which results in its increased interaction with upstream regulators and consequent kinase priming and activating phosphorylation.

**Steric effects**
A phenomenon arising as a result of the fact that each residue and its atoms occupy a certain amount of space in a defined conformation.

**Residence time**
The period for which the target kinase is occupied by the small-molecule inhibitor.

**Off-rate**
The rate of dissociation of the small-molecule inhibitor from the kinase.

**Cross-resistance**
The development of tumour resistance to a potential therapy after treatment of a patient with a different therapeutic agent.

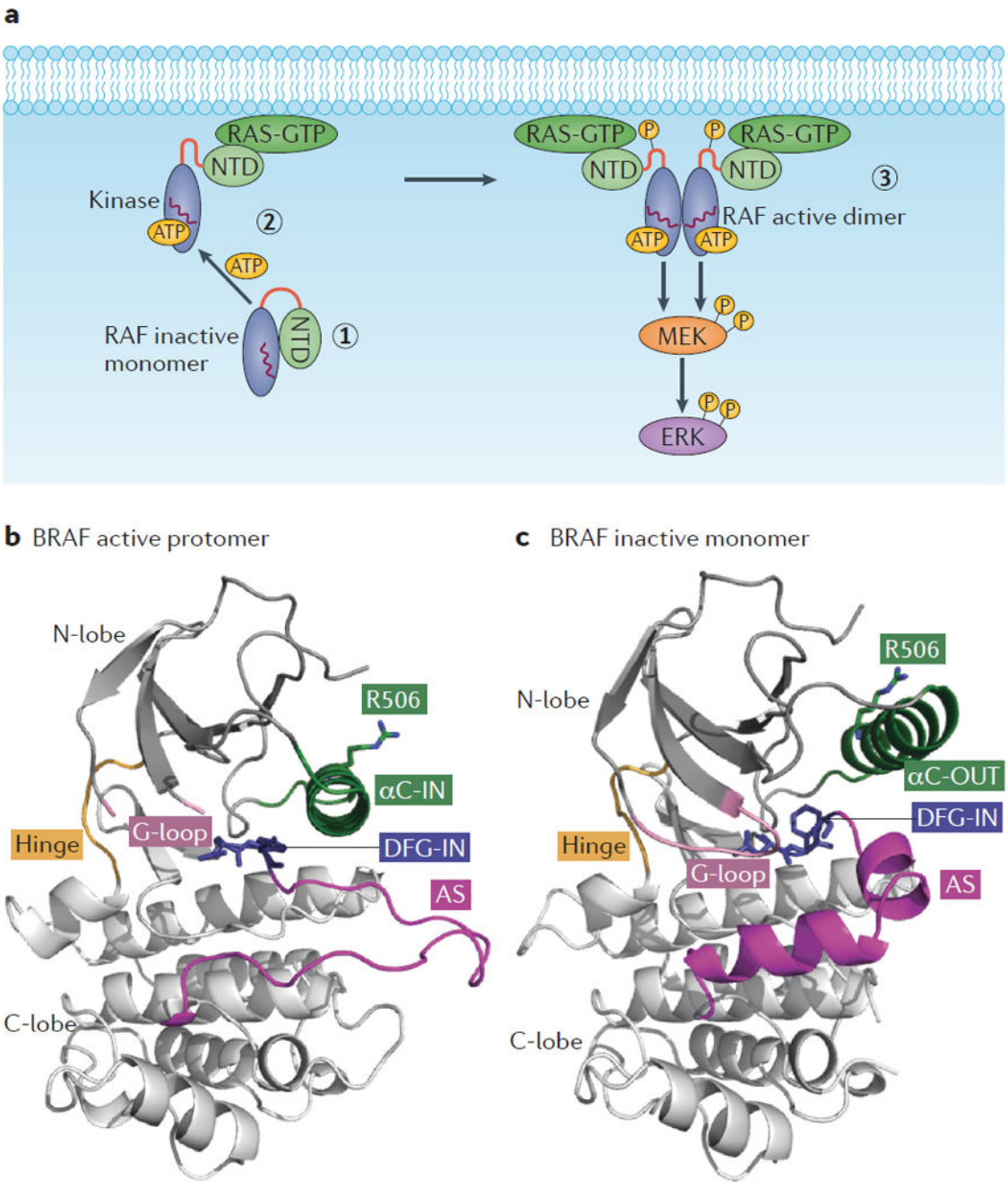



**Figure 1. RAF activation**

**a** | Schematic showing canonical RAF activation. In conditions of low RAS-GTP, RAF is monomeric and inactive in the cytosol due to intramolecular interaction between the N-terminal domain (NTD; regulatory) and the C-terminal (kinase) domain (1). Upregulation of RAS-GTP promotes the formation of the RAF–RAS-GTP complex in the membrane due to the high affinity of RAS-GTP for the RAS-binding domain (RBD) present in the NTD of RAF (2). This step is followed by activating phosphorylation and dimerization for full RAF activation (3). αC-helix indicated by the red wavy line in the kinase domain. **b** | Ribbon

representation of one protomer of the active BRAF dimer (Protein Data Bank (PDB) ID: 4MNE). The kinase domain consists of N and C terminal lobes linked through a short flexible hinge. **c** | Ribbon representation of the inactive and monomeric BRAF kinase domain (PDB ID: 4RZV). In parts **b** and **c**, note the different positions of the αC-helix (in green) and of the activation segment (AS, magenta), which highlight the movement of the αC-helix from the OUT to the IN position and the unfolding of the AS upon RAF activation. The R506 residue side chain in the IN (active) and the OUT (inactive) positions and the conformation of the glycine-rich loop (G-loop) and DFG motif in the active and inactive form are also depicted.

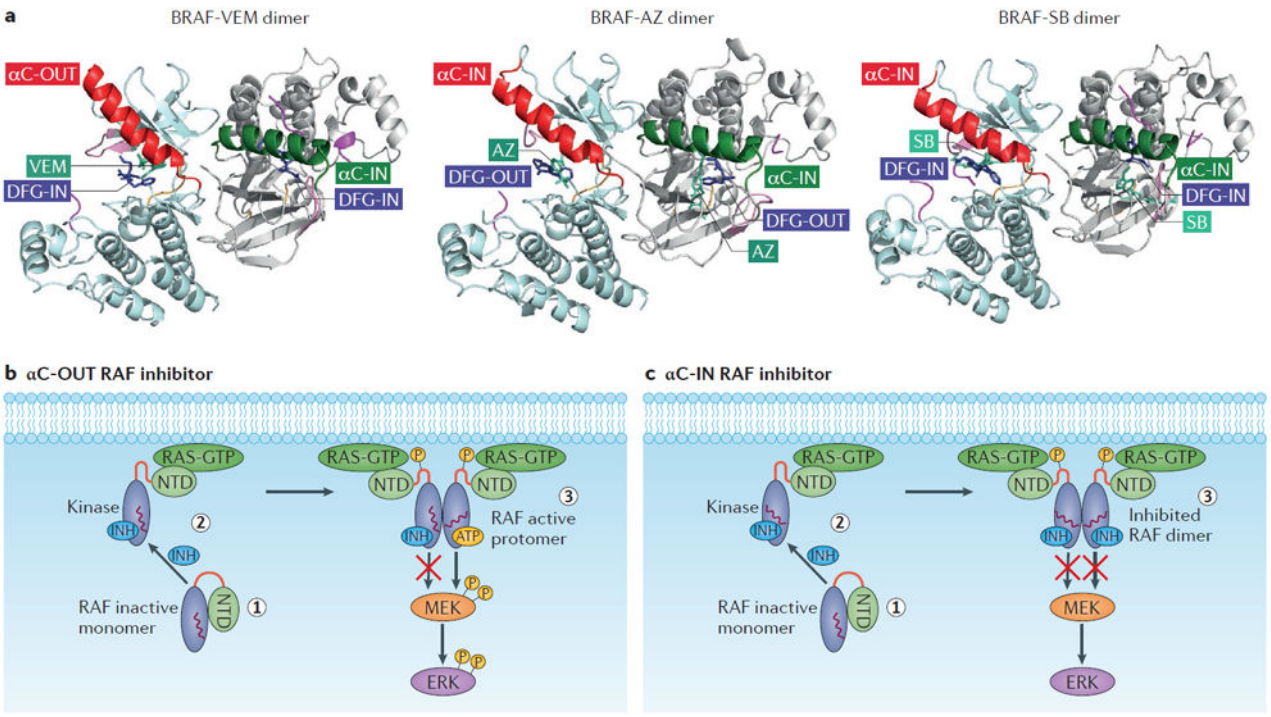



**Figure 2. Mechanism of action of RAF inhibitors**

**a** | Structural features of representative RAF inhibitors bound to a BRAF dimer. The three structural types of RAF inhibitor bound to BRAF are shown as ribbon representations: Vemurafenib (VEM), a type $I^{1/2}$ inhibitor (αC-OUT/DFG-IN), AZ628 (AZ), a type II inhibitor (αC-IN/DFG-OUT), and SB590885 (SB), a type I inhibitor (αC-IN/DFG-IN). Vemurafenib and other type $I^{1/2}$ inhibitors stabilize the αC-helix (red) of the first BRAF protomer to the OUT position and the αC-helix (green) of the second BRAF protomer to the IN position (Protein Data Bank (PDB) ID: 3OG7). AZ628 and other type II inhibitors stabilize the αC-helix of the first BRAF protomer (red) and the αC-helix of the second BRAF protomer (green) to the IN position (PDB ID: 4RZW). SB590885 and other type I inhibitors stabilize both the αC-helix of the first BRAF protomer (red) and the αC-helix of the second BRAF protomer (green) to the IN position (PDB ID: 2FB8). AZ628 and other type II inhibitors induce the DFG motif to an OUT conformation, while other inhibitors of the type I and $I^{1/2}$ groups maintain the DFG in an IN conformation. **b** | Schematic showing the αC-OUT RAF inhibitor-induced paradoxical RAF pathway activation and negative allostery. Most αC-OUT RAF inhibitors stabilize the αC-helix (indicated by the red wavy line in the kinase domain) in the overall OUT position (1). However, the C-terminal αC-helix residue R506 is displaced into the IN position, which promotes the interaction of RAF with RAS-GTP (2). Subsequently, RAF is primed through phosphorylation and dimerization. RAF inhibitor binding to one protomer and stabilization of the αC-helix in the OUT position sterically prevents inhibitor binding to the second monomer (negative allostery), leading to paradoxical pathway activity (3). **c** | Schematic showing αC-IN RAF inhibitor-induced RAF activation and effective dimer inhibition. By stabilizing the αC-helix in the IN position (1), αC-IN RAF inhibitors promote the formation of the RAF–RAS–GTP complex (2). This is followed by RAF phosphorylation and dimerization. Due to low

negative allostery, RAF inhibitors belonging to this group effectively bind and inhibit both protomers in the RAF dimer (3). INH, inhibitor; NTD, N-terminal domain.

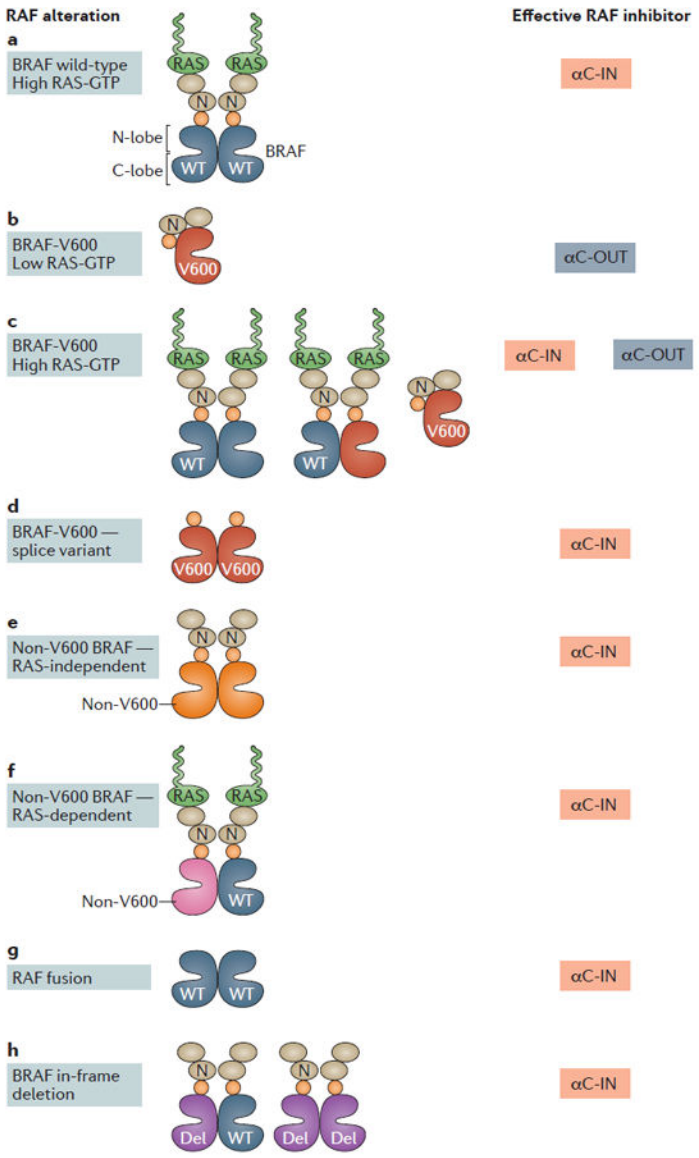



**Figure 3. Categories of RAF alterations found in tumours and the types of RAF inhibitor predicted to be effective against them**

**a** | In the cellular context of RAS-activated wild-type (WT) BRAF, BRAF, CRAF and ARAF form active homodimers and heterodimers that are resistant to αC-OUT RAF inhibitors. αC-IN RAF inhibitors are predicted to potently inhibit RAF dimers, although their effectiveness will be limited due to concurrent formation of active RAF dimers by the inhibitor. **b** | Mutant BRAF-V600 proteins in the absence of RAS-GTP signal as a monomer and are potently inhibited by either αC-OUT or αC-IN inhibitors. In this context, αC-OUT inhibitors are predicted to be more effective treatments due to their wide therapeutic window. **c** | In the context of tumours expressing BRAF-V600 in the presence of active RAS, ERK is activated by both BRAF-V600E monomers and RAF dimers (homodimers and heterodimers of BRAF-V600E, WT BRAF, CRAF and ARAF). A combination of a αC-OUT inhibitor with a αC-IN RAF inhibitor is predicted to result in effective RAF inhibition

in the tumour while retaining a wide therapeutic window. **d** | A BRAF-V600E splice variant that lacks part of the N-terminal domain and constitutively dimerizes has been identified in tumours that have developed clinical resistance to αC-OUT RAF inhibitors. In this context, ERK is activated by BRAF-V600E homodimers, and αC-IN RAF inhibitors may be an effective therapeutic option. **e** | For tumours expressing mutant BRAF proteins other than BRAF-V600 that signal as RAS-independent dimers, αC-IN RAF inhibitors may be an effective therapeutic option. **f** | For tumours expressing mutant BRAF proteins other than V600 that require RAS activity, αC-IN RAF inhibitors may be an effective therapeutic option. **g** | Fusion transcripts of various genes with the catalytic domain of either WT BRAF or, in some cases, CRAF, lack the N-terminal domain that prevents RAF dimerization, and their protein products thus signal as RAS-independent RAF dimers. For patients whose tumours depend on such fusion proteins, αC-IN RAF inhibitors may be an effective therapeutic option. **h** | In-frame deletions in BRAF that stabilize the αC-helix in the active (IN) position have been found in tumours. For patients with such tumours, αC-IN RAF inhibitors may be an effective therapeutic option. C-lobe, C-terminal lobe; N, N-terminal domain; N-lobe, N-terminal lobe; Del, in-frame deletion; αC-IN, αC-helix-IN RAF inhibitor; αC-OUT, αC-helix-OUT RAF inhibitor.

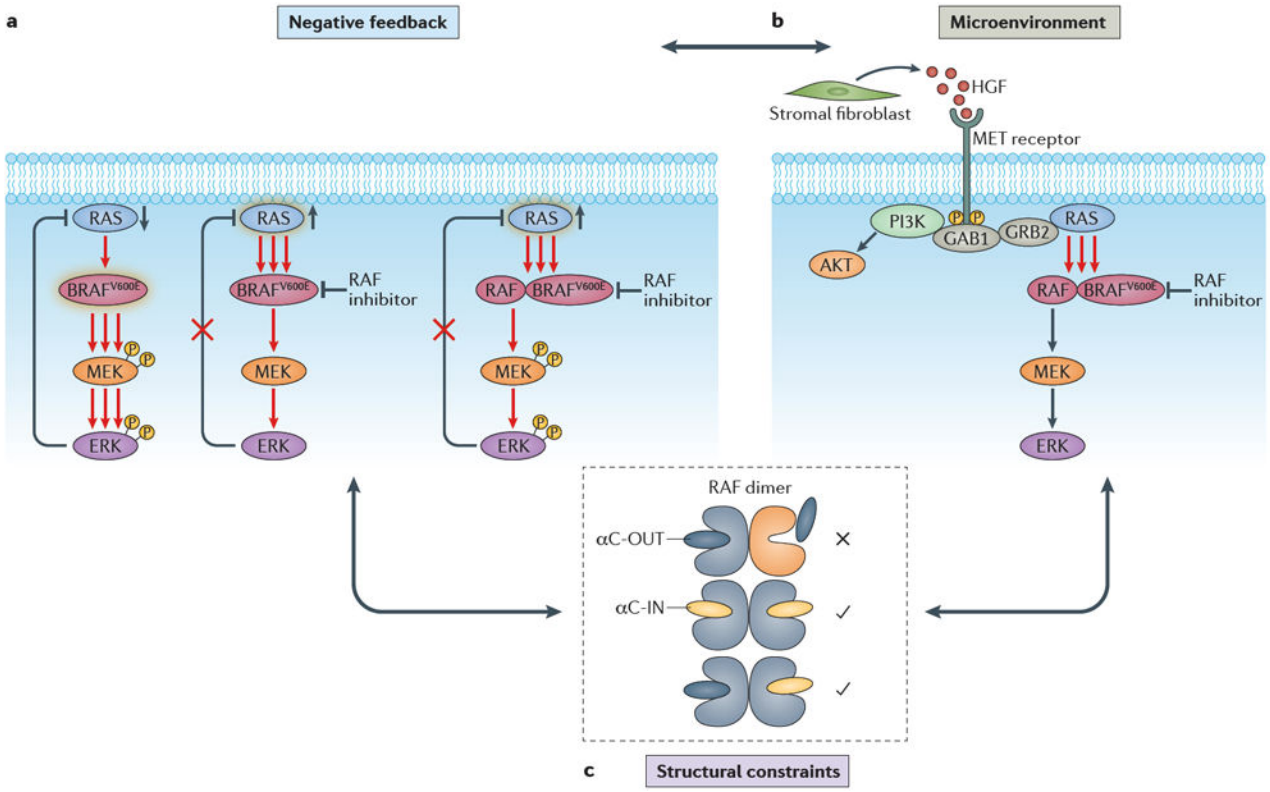
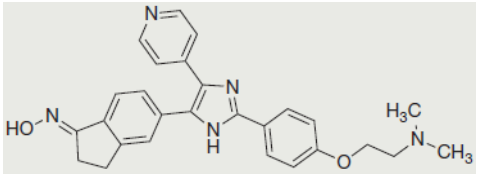
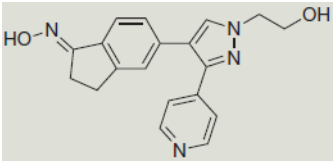
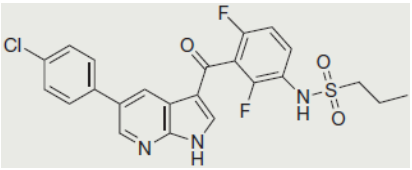
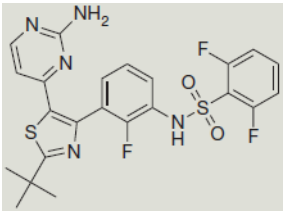


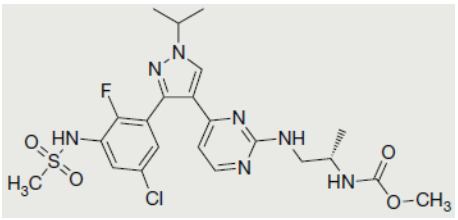
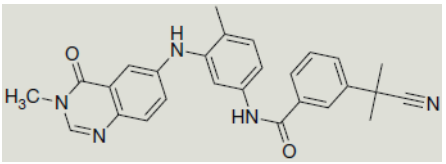
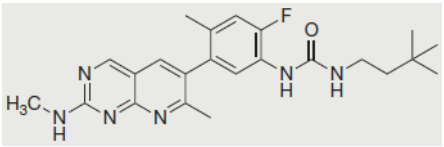
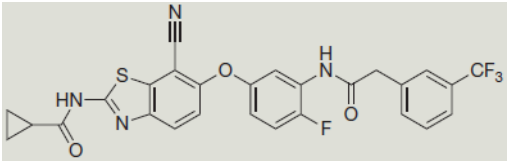
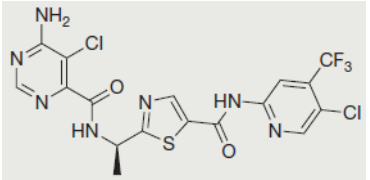

**Figure 4. The interplay of mechanisms of adaptive response to RAF inhibitors**

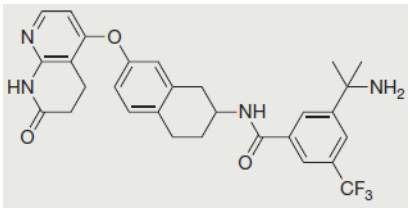
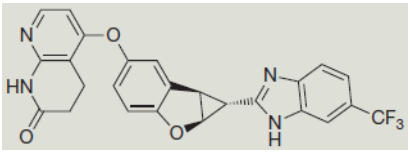
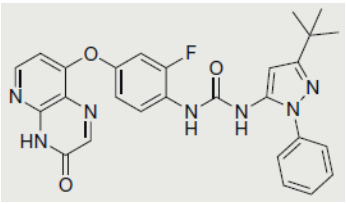
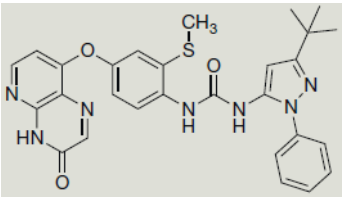
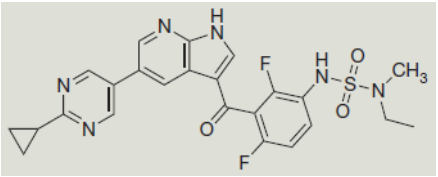
**a** | Relief of negative feedback. At steady state, hyperactivated ERK downstream of mutant BRAF suppresses RAS due to negative feedback. Addition of a RAF inhibitor results in relief of negative feedback and RAS activation, which in turn promotes increased flux through ERK signalling and RAF dimerization[41,73,74]. Activation indicated by a green halo around the protein. **b** | Pathway reactivation due to paracrine signalling by components of the tumour microenvironment (TME). Stromal cells in the TME release growth factors (exemplified here by hepatocyte growth factor (HGF)) and cytokines, which promote resistance to RAF inhibitors by activating parallel survival pathways, such as PI3K signalling, or by activating RAS[75-77]. **c** | Structural constraints. RAF dimerization promotes an adaptive response to αC-OUT RAF inhibitors due to negative allostery for inhibitor binding to the second protomer of a RAF dimer when the first is occupied by inhibitor. αC-IN RAF inhibitors are predicted to overcome this resistance mechanism by binding with similar affinity to both protomers of the RAF dimer[41]. All of these mechanisms of adaptive response are interconnected and converge upon structural constraints for inhibitor binding. Relief of negative feedback promotes upstream receptor tyrosine kinase activation, which may be further enhanced by growth factors and cytokines released in the TME. Both mechanisms promote increased flux in the pathway and levels of active RAF molecules. They also promote RAF dimerization, which prevents effective RAF inhibition by current clinical αC-OUT RAF inhibitors due to negative allostery. GAB1, growth factor receptor-bound protein 2-associated binding protein 1; GRB2, growth factor receptor-bound protein 2.

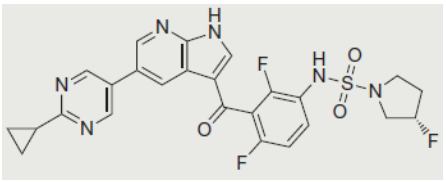
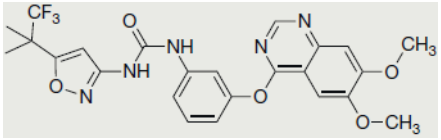
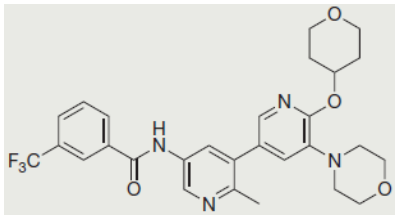
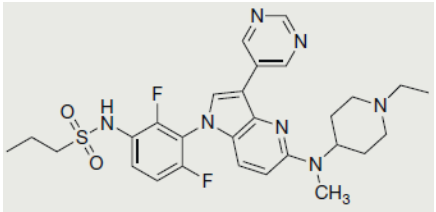
**Table 1**

Select RAF inhibitors and their stage of development

| RAF inhibitor | Class | Stage of development | Chemical structure | Clinical trial | Ref. |
|---|---|---|---|---|---|
| SB590885 | αC-IN/DFG-IN | Preclinical |  | — | — |
| GDC0879 | αC-IN/DFG-IN | Preclinical |  | — | — |
| Vemurafenib | αC-OUT/DFG-IN | Approved for BRAF-V600E melanoma |  | — | — |
| Dabrafenib | αC-OUT/DFG-IN | Approved for BRAF-V600E/K melanoma |  | — | — |

| RAF inhibitor | Class | Stage of development | Chemical structure | Clinical trial | Ref. |
|---|---|---|---|---|---|
| LGX818 | αC-OUT/DFG-IN | Phase III |  | NCT01909453 and NCT02928224 | 104,125 |
| AZ628 | αC-IN/DFG-OUT | Preclinical |  | — | — |
| LY3009120 | αC-IN/DFG-OUT | Phase I |  | NCT02014116 | 112 |
| TAK632 | αC-IN/DFG-OUT | Preclinical |  | — | — |
| MLN2480 (TAK580) | NA (presumably αC-IN/DFG-OUT) | Phase I |  | NCT01425008, NCT02723006 and NCT02327169 | 108-110 |

| RAF inhibitor | Class | Stage of development | Chemical structure | Clinical trial | Ref. |
|---|---|---|---|---|---|
| BGB659 | NA (presumably αC-IN/DFG-OUT) | Preclinical |  | — | — |
| BGB283 | αC-IN/DFG-OUT | Phase I |  | NCT02610361 | 114 |
| CCT196969 | NA (presumably αC-IN/DFG-OUT) | Preclinical |  | — | — |
| CCT241161 | NA (presumably αC-IN/DFG-OUT) | Preclinical |  | — | — |
| CCT3833 (BAL3833) | NA (presumably αC-IN/DFG-OUT) | Phase I | NA | NCT02437227 | 107 |
| PLX7904 | αC-OUT/DFG-IN/R506-OUT | Preclinical |  | — | — |

| RAF inhibitor | Class | Stage of development | Chemical structure | Clinical trial | Ref. |
|---|---|---|---|---|---|
| PLX8394 | αC-OUT/DFG-IN/R506-OUT | Phase I/II |  | NCT02428712 | 116 |
| HM95573 | NA | Phase I | NA | NCT03118817 and NCT02405065 | 121,122 |
| CEP32496 (also known as RXDX-105) | NA | Phase I |  | NCT01877811 | 124 |
| LXH254 | NA | Phase I | NA | NCT02607813 | 115 |
| RAF709 | αC-IN/DFG-OUT | Preclinical |  | — | — |
| BI 882370 | αC-OUT/ DFG-OUT | Preclinical |  | — | — |

NA, not available.