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Review

Challenges and Opportunities in the Crusade of BRAF Inhibitors: From 2002 to 2022

Ankit Kumar Singh, Pankaj Sonawane, Adarsh Kumar, Harshwardhan Singh, Vladislav Naumovich, Prateek Pathak, Maria Grishina, Habibullah Khalilullah, Mariusz Jaremko, Abdul-Hamid Emwas, Amita Verma, and Pradeep Kumar*

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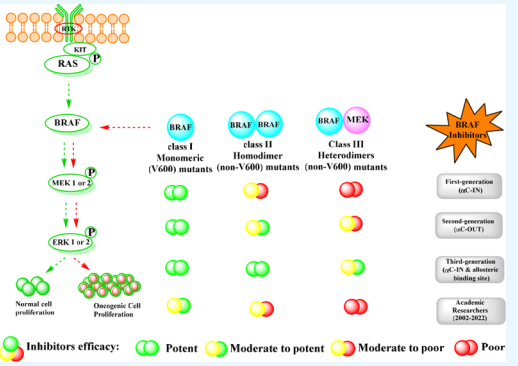
ABSTRACT: Serine/threonine-protein kinase B-Raf (BRAF; RAF = rapidly accelerated fibrosarcoma) plays an important role in the mitogen-activated protein kinase (MAPK) signaling cascade. Somatic mutations in the BRAF gene were first discovered in 2002 by Davies et al., which was a major breakthrough in cancer research. Subsequently, three different classes of BRAF mutants have been discovered. This class includes class I monomeric mutants ($BRAF^{V600}$), class II BRAF homodimer mutants (non-V600), and class III BRAF heterodimers (non-V600). Cancers caused by these include melanoma, thyroid cancer, ovarian cancer, colorectal cancer, nonsmall cell lung cancer, and others. In this study, we have highlighted the major binding pockets in BRAF protein, their active and inactive conformations with inhibitors, and BRAF dimerization and its importance in paradoxical activation and BRAF mutation. We have discussed the first-, second-, and third-generation drugs approved by the Food and Drug Administration and drugs under clinical trials with all four different binding approaches with DFG-IN/OUT and αC-IN/OUT for BRAF protein. We have investigated particular aspects and difficulties with all three generations of inhibitors. Finally, this study has also covered recent developments in synthetic BRAF inhibitors (from their discovery in 2002 to 2022), their unique properties, and importance in inhibiting BRAF mutants.



1. INTRODUCTION

The rat sarcoma (RAS)/rapidly accelerated fibrosarcoma (RAF)/mitogen extracellular kinase (MEK)/extracellular signal-regulated kinase (ERK) or mitogen-activated protein kinase (MAPK) signaling pathway is a crucial intracellular signaling pathway,[1] responsible for intracellular signal transduction, namely, acute hormone responses, embryogenesis, cell differentiation, and apoptosis involved in the control of cell growth, proliferation, migration, and survival.[2] By acting downstream of receptor tyrosine kinases, the MAPK pathway regulates cell fate decisions. Under physiological conditions, MAPK activation occurs via extracellular binding of growth factors to receptor tyrosine kinases,[3] at the cell surface, leading to phosphorylation and activation of RAS proteins by guanosine-5′-triphosphate (GTP)-bound.[4] RAF, a downstream effector of RAS, is a serine-threonine-specific protein kinase that stimulates MEK, which in turn activates ERK.[5] Activation of ERK results in a growth-promoting and transforming signal (Figure 1).[6,7]

The RAF kinase family includes three members of the MAPK pathway: ARAF, BRAF, and CRAF. BRAF is the major activating kinase,[8] and its activation occurs by binding to its RAS-binding domain.[9] It encodes a cytoplasmic serine/threonine kinase and is a major regulator of the RAS pathway (RAS → RAF → MEK → ERK → MAP kinase).[10,11] BRAF is essential for regulation of a variety of cellular processes, including growth, proliferation, survival, and migration.[12,13]

The discovery of BRAF mutations was a major breakthrough in cancer research. Somatic mutations in BRAF were first discovered in 2002 by Davies et al.[14] BRAF is among the most frequently mutated oncogenes in human cancers.[15] One of the most studied pathways, with over 90% mutations, is the RAS > RAF > MEK > ERK (MAPK) pathway.[16] BRAF mutations occur to varying degrees in human cancers, including approximately 70−90% in melanoma, 5−20% in colon cancer,[17,18] 30−50% in thyroid cancer,[19] 5−30% in ovarian cancer,[20] 1−4% in nonsmall cell lung cancer, and 1−3% in hairy cell leukemia and Langerhans cell histiocytosis (Figure 2).[21]

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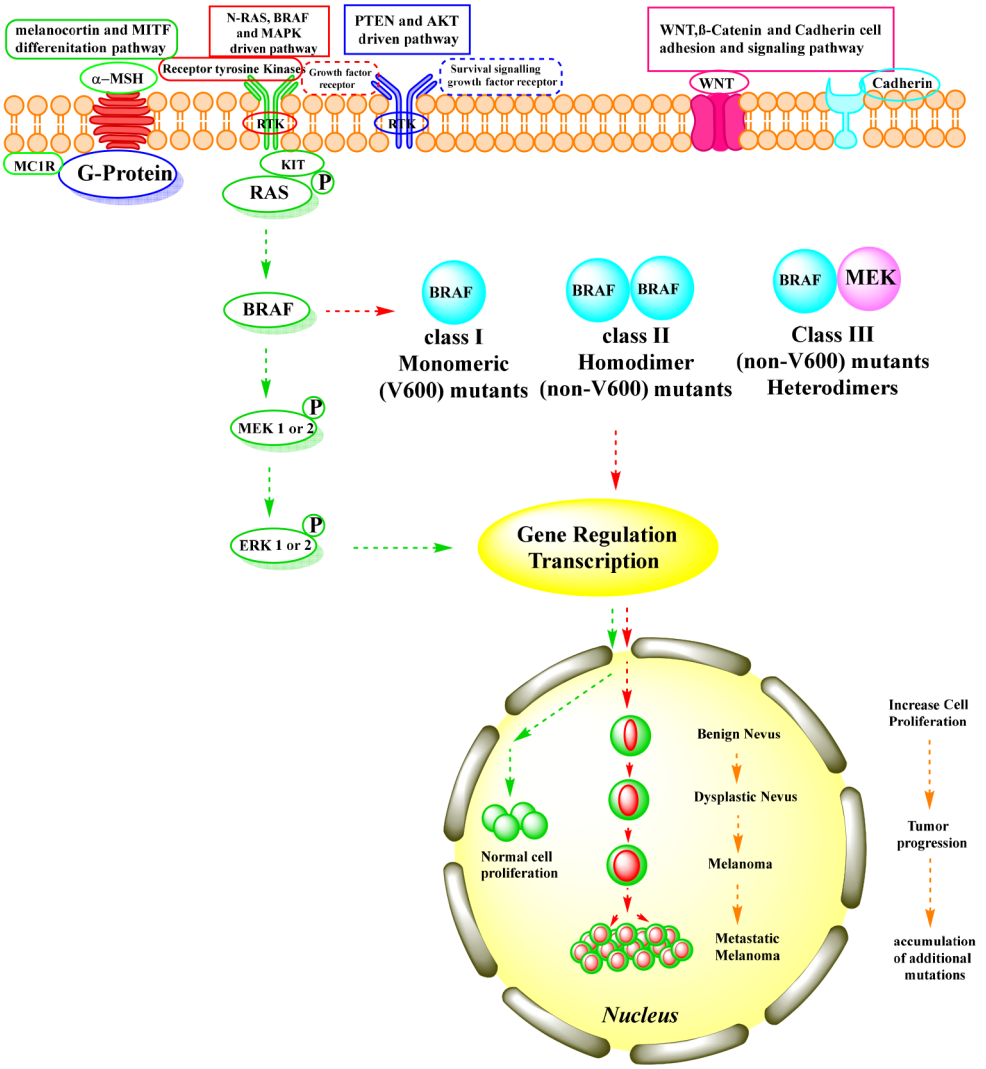

Figure 1. BRAF signaling pathway. (green arrow) Normal BRAF pathway. (red arrow) Oncogenic BRAF pathway.

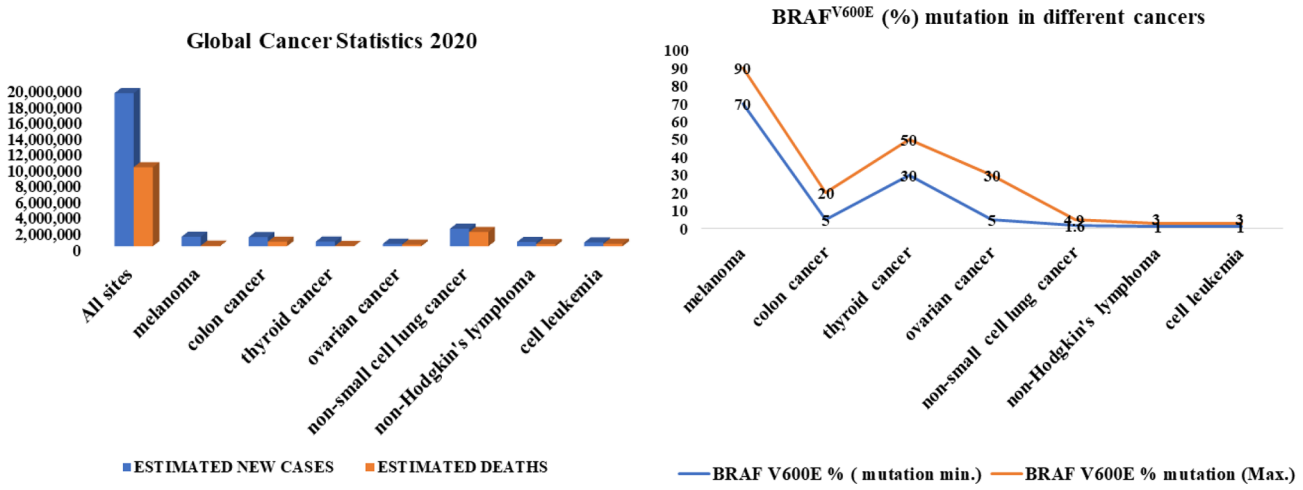

Figure 2. Global cancer statistics for new cases and deaths for 2020 and $BRAF^{V600E}$ (%) mutation in different cancer types.

All BRAF mutations are located in the kinase domain, either in the glycine-rich loop encoded by exon 11 or in the activation sequence encoded by exon 15.[14,22] The BRAF gene has a variety of activating mutations that have been discovered and classified into three classes. The monomeric $BRAF^{V600}$ codon mutation is classified as class I. In the RAS/RAF/MEK/ERK pathway, this mutation occurs in an RAS-independent manner.[23] The $BRAF^{V600}$ gene mutation $BRAF^{V600E}$ is the most common mutation 90%[24] at position 600 with a single point mutation (Val600 → Glu) in which the polar, hydrophilic

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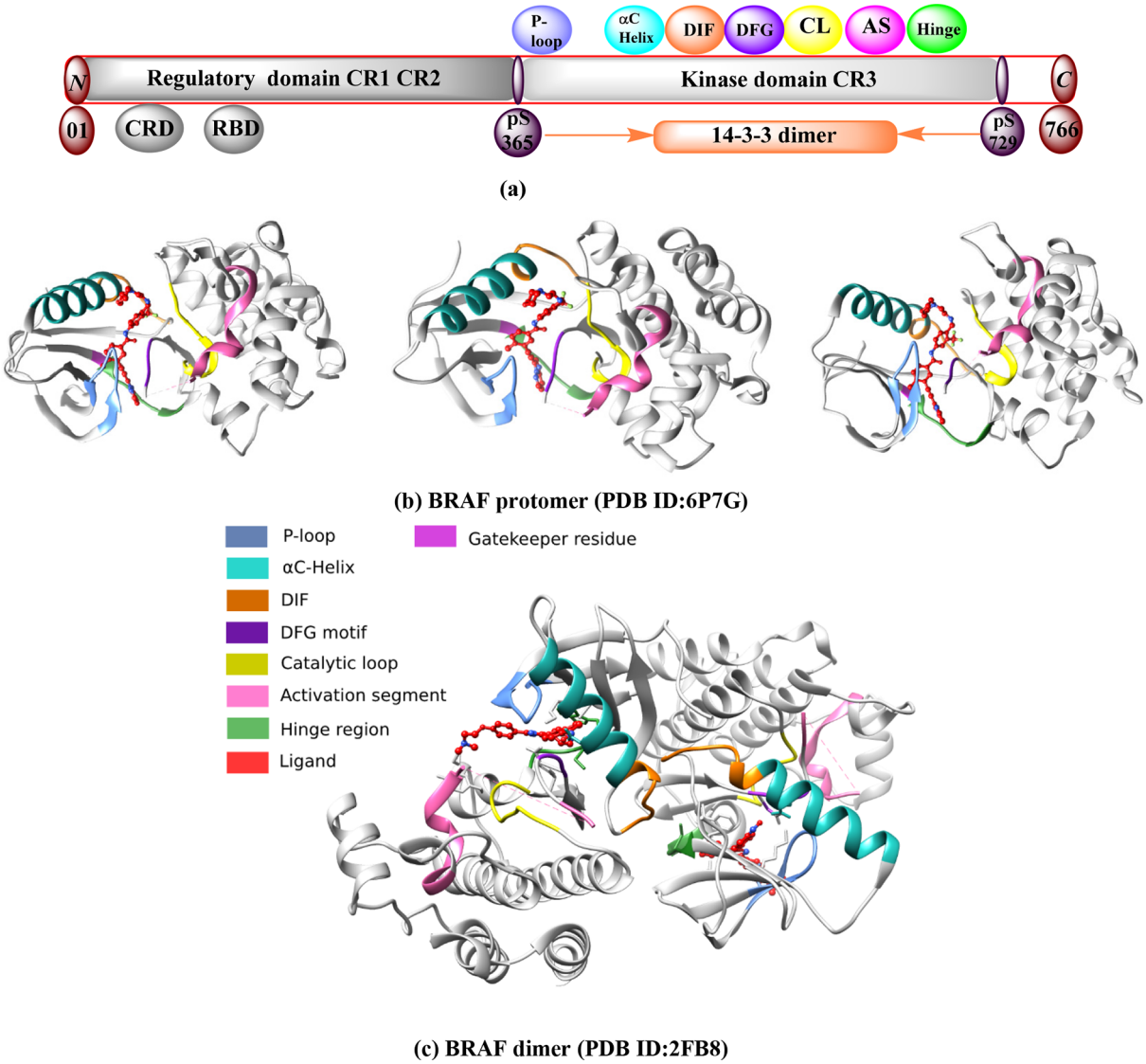

Figure 3. BRAF protein structure and binding pocket. (a) Regulatory (CR1 & CR2) and kinase (CR3) domain between N and C-terminal in BRAF kinase protein. (b) BRAF protomer (PDB ID:6P7G) with different orientations. (c) BRAF dimer (PDB ID:2FB8). Different binding pockets (p-loop, αC-helix, DIF, DFG, catalytic loop, activation segment, and hinge region) are labeled in specific colors.

glutamic acid replaces the hydrophobic valine.[25] This breaks the hydrophobic link between the A-loop and the P-loop, resulting in an active conformation.[26] The constitutive kinase activity of $BRAF^{V600E}$ is 500 times higher than that of wild-type BRAF kinase.[27] Another type of mutation is $BRAF^{V600WT}$, with $BRAF^{V600K}$ (10%) being the second most common mutation, where valine is replaced by lysine.[28] Rarer codon $BRAF^{V600}$ mutations are $BRAF^{V600R}$, $BRAF^{V600D}$ (6−3%), and $BRAF^{V600E2,V600M,V600G}$ (<1%).[29] The class II includes non-V600 mutants and is regulated by mutant BRAF homodimers without involvement of RAS. Here, L597 and K601 were found in the activation segment, whereas G464 and G469 were found in the glycine-rich region of BRAF. Class III involves non-V600 mutants and is regulated by mutant BRAF heterodimers involving the RAS mutant. In this, D287, V459L, G466 V, S467, G469, N581, D594, F555, and G596 were detected. In BRAF gene mutations, the classes II and III are very rare codon mutations.[23,30]

Statistics of new cases and deaths in all cancers worldwide for 2020 and details of specific cancers affected by the $BRAF^{V600E}$ mutation are shown in (Figure 2).

2. CURRENT INSIGHT BRAF PROTEIN

2.1. BRAF's Skeletal System and Binding Pocket. The BRAF protein consists of 766 amino acid sequences.[31,32] It has two lobes, the N-terminal and the C-terminal lobes. These two lobes consist of three conserved regions (CRs): CR1 (R150−290), CR2 (R360−375), and CR3 (R457−717). CR1 and CR2 are regulatory domains.[33] CR1 contains the RAS-binding domain (RBD) (R155−227) and the cysteine-rich domain (CRD) (R234−280).[34,35] CR1 interacts with both RAS and membrane phospholipids. CR1 acts as an autoinhibitor of the BRAF kinase domain (CR3) and ensures that signal transduction is controlled. CR2 is a serine/threonine-rich domain that serves as a flexible hinge between CR1 and CR3 and contains a binding site for the 14−3−3 protein.[35,36] CR3 is the catalytic protein kinase domain located near the C-terminus [Figure 3a]. The N-terminus of CR3 is involved in adenosine

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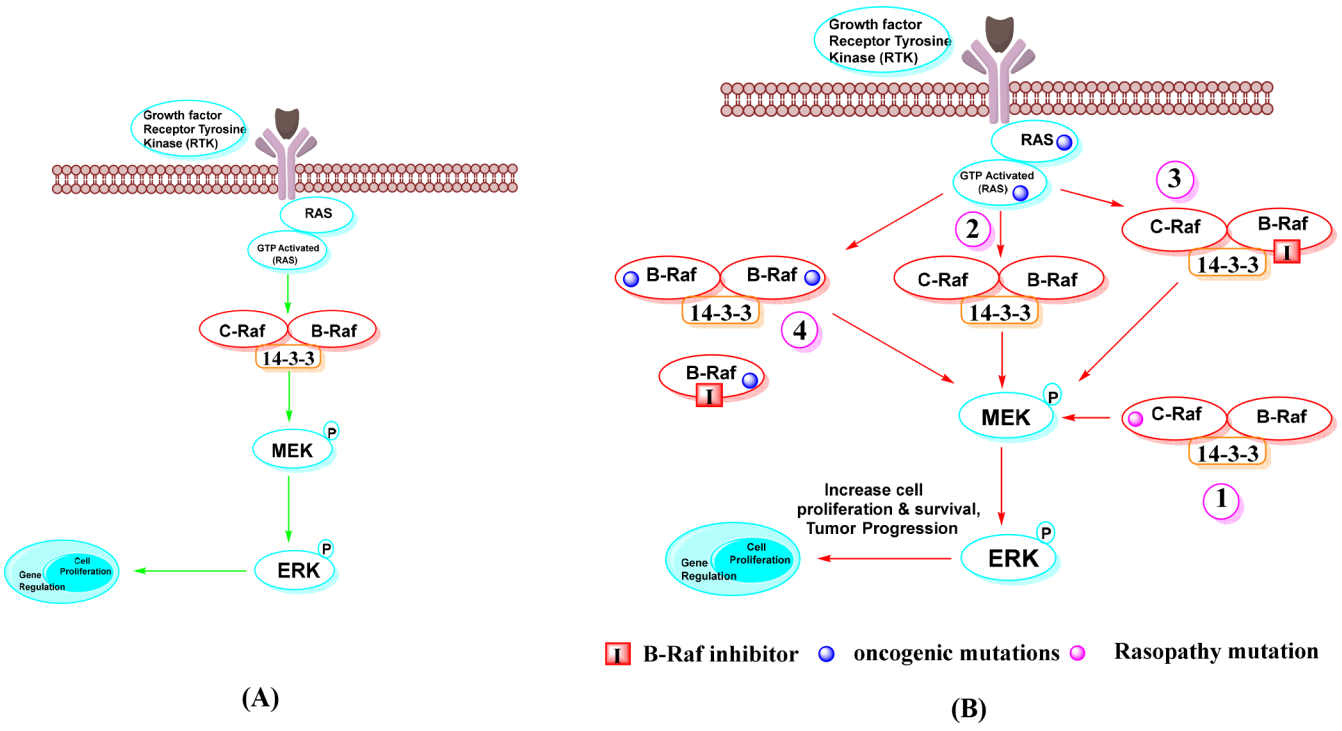

Figure 4. Dimerization of Raf in cell signaling. Note: (A) In the normal RAS-dependent signaling pathway, Raf dimerization is required for Raf kinase activation and signaling to MEK for further cell proliferation and regulation. (B) In oncogenic states, it is required for MEK/ERK signaling, which is upregulated by Raf dimerization. These include: (1) mutant c-Raf protein with b-Raf in dimerization impaired kinase activity from normal to oncogenic, (2) RTKs and RasGTPases induced by mutation, (3) in the context of active Ras, treatment with ATP-competitive Raf inhibitors, and (4) Raf inhibitor resistance is mediated by self-homodimerizing $BRAF^{V600E}$ splice variants.

triphosphate (ATP) binding. It primarily comprises an antiparallel β-sheet structure, anchors, and a glycine-rich ATP-phosphate binding loop (P-loop) (R464−471) and an αC-helix (R492−504). Through the activation segment, the P-loop region is critical for stabilizing ATP binding and maintaining an inactive BRAF conformation. The C-terminal of CR3 contains a hinge region/adenine region (R530−535), catalytic loop (R574−581), DFG motif [Asp-Phe-Gly(R594−595−596)], and an activation section (R594−623). In the CR3 domain, the ATP-phosphate binding loop, DFG motif, and activation segment are the major active binding sites.[37−39] The ATP pocket has several binding regions that interact with inhibitors. These are an adenine region (similar to the hinge region), a sugar region (ATP ribose pocket), type I and II hydrophobic regions, and a solvent-accessible region that is also part of the ATP region but is not occupied. At the C-terminus, the dimerization interface (DIF) (R504−511) is another binding site for the 14−3−3 protein.[36] The end of CR3 facilitates the binding of substrate proteins. This occurs through its catalytic loop, which facilitates the transfer of phosphate from ATP to BRAF substrates [Figure 3b,c].[32,40] The gatekeeper (catalytic cleft between the N- and C-termini) plays an important role by controlling the access of ligands to a hydrophobic pocket deep in the active site that is not in contact with ATP, which affects the binding and specificity of inhibitors.[41]

2.2. Importance of BRAF Dimerization and Its Challenges. Recent studies have shown that Raf dimerization is required for normal Ras-dependent activation of Raf kinase in various cellular activities.[42] The active conformation of BRAF is facilitated by dimerization.[43] It was discovered that dimerization of BRAF kinases involves insights into a core cluster of residues known as the dimer interface (DIF) [(R504−511) in BRAF], which plays a critical role in dimer formation.[8] DIF is located at the tail end of the αC-helix in the BRAF kinase protein. Many of the ATP-competitive inhibitors promote BRAF dimerization in an RAS-dependent manner. BRAF dimerization can be homo- or heterodimerized.[44] The 14−3−3 domain protein in DIF is responsible for BRAF dimerization. The two-protein structure of BRAF−BRAF in the 14−3−3 complex in DIF is responsible for homodimer, and BRAF-MEK1−14−3−3 complex is heterodimer. Two BRAF serine sites pS729 and Ps365 open monomers in the 14−3−3 protein complex, promote formation of active BRAF dimers, and link them.[36] Dimerization is enhanced by Ras and is subject to negative feedback regulation by ERK.[45]

The structure of an active BRAF kinase in dimerization is that of a side-by-side dimer, in which only one partner needs to be catalytically active.[45] Drugs that inhibit BRAF kinase can bind to a protomer of the dimer complex (homodimers and heterodimers of BRAF) and inhibit its ATP-binding site; however, this binding enhances the activity of the protomer (in the absence of drug), leading to the induction of an active conformation.[46] Although recent BRAF dimer structures have shown that the 14−3−3 dimer can bind to two kinase domain protomers simultaneously, it is unclear whether both protomers are catalytically active.[47]

The RBD:14−3−3 interface has a dual function, contributing first to RAF autoinhibition and second to the full spectrum of RAS-RBD interactions.[47] There are two types of auto-inhibitory mechanisms that must be overcome in BRAF activation. The first is 14−3−3-assisted locking of the N- and C-terminal portions, and the second is destabilization of the inactive conformation of the kinase domain itself.[46] BRAF

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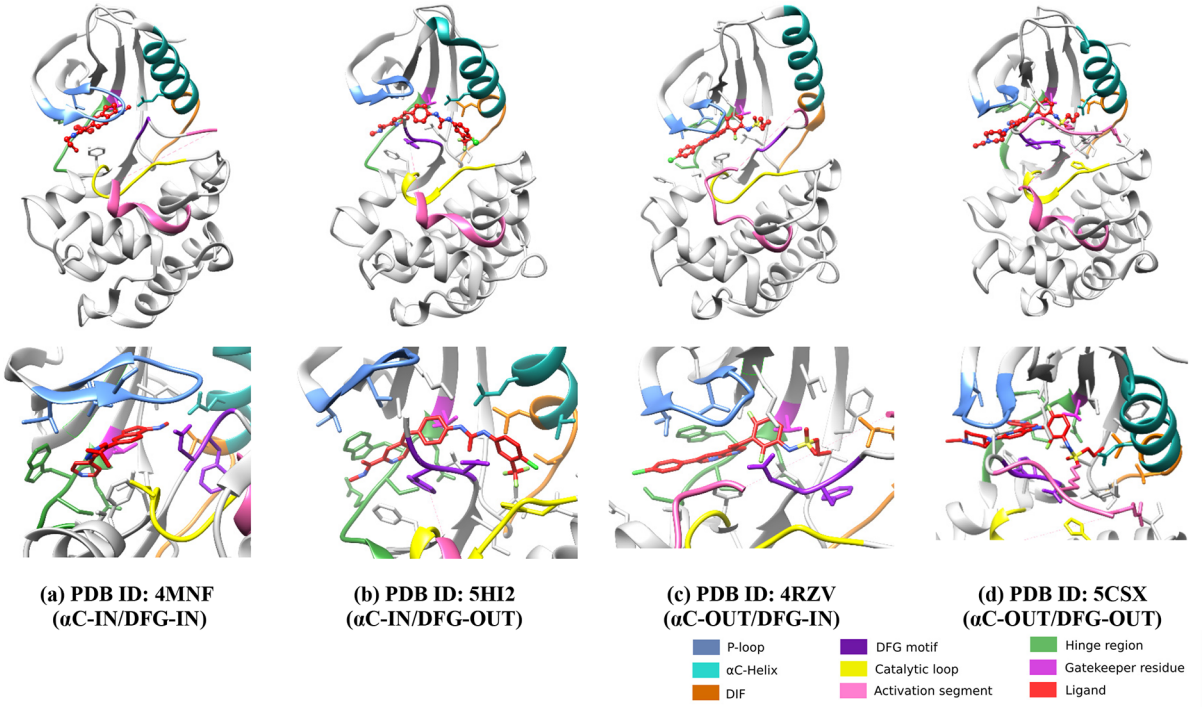

Figure 5. On the basis of DFG (purple color) and αC (cyan color) movement, four different binding conformations are shown. **(a) PDB ID: 4MNF** (GDC-0879); type I inhibitors (αC-IN/DFG-IN) **(b) PDB ID: 5HI2** (Sorafenib); type II inhibitors (αC-IN/DFG-OUT) **(c) PDB ID: 4RZV** (Vemurafenib); type $I_{1/2}$ or type III (αC-OUT/DFG-IN) **(d) PDB ID: 5CSX** (BI 882370); type$_{I/II}$ or type IV (αC-OUT/DFG-OUT).

dimerization is also associated with multiple phosphorylation events. RAF dimers may have an allosteric function in facilitating activation loop autophosphorylation. The autophosphorylation of the P-loop of RAF is inextricably linked to the catalytic activity of RAF.[48] The dimer-promoting potential of BRAF inhibitors may be associated with some "paradoxical" activation. Paradoxical triggers may enhance concerted movements of the αC-helix and A-loop regions.[49] As mentioned earlier, the class II mutation is responsible for the homodimer, and the class III mutation is responsible for the heterodimer. Rajakulendran et al. suggested that BRAF mutations G450W, F488A, M489W, and Y538F at the dimerization interface inhibit dimer formation.[46] BRAF dimerization contributes to the pathogenic effect of disease-associated mutant Raf proteins and, when activated, exhibits similar activity to the constitutively active V600E mutant. It has also been shown to influence treatment response and disease progression in individuals receiving BRAF inhibitors.[50] Raf dimerization and its role in normal and oncogenic states in cell signaling is shown in Figure 4.

2.3. Concept of Inactive and Active Conformation of BRAF. BRAF protein switches from inactive to active conformation and is also important for kinase activation. Several regulatory elements play key roles in this process. The inactive conformation of BRAF is characterized by the position DFG-OUT/αC-OUT and is also known as the closed conformation. The active conformation is characterized by the position DFG-IN/αC-IN and is also referred to as the open conformation.[51−53] In BRAF protein kinases, the conformation of the conserved DFG motif containing a catalytic [Asp-Phe-Gly (594−595−596)] residue is known to be critical for selective binding of many kinase inhibitors.[54]

In the DFG-OUT conformation, BRAF shows a reverse orientation. The DFG residue [Asp (594)] oscillates and is displaced from the active site, occupying part of the ATP-binding site. The Mg^{2+} of the DFG motif is not bound to ATP, facilitating ATP c-phosphate transfer as the catalytic cleft of BRAF becomes inaccessible to ATP. The glycine-rich loop and activation segment are close to each other due to hydrophobic interactions.[55,56] The DFG-OUT conformation has created an ATP-binding site and an adjacent hydrophobic pocket. In this conformation, the DFG residue moves from the ATP-binding site to the hydrophobic pocket, which is known as allosteric movement extending outward from the ATP-binding site. In the DFG-OUT conformation, the inhibitors bind to the allosteric site (hydrophobic pocket, near the ATP-binding pocket).[54]

In the DFG-IN conformation, the DFG residue [Asp(594)] is in the active site, and the catalytic cleft of BRAF becomes accessible to ATP, so that the Mg^{2+} of the DFG motif is bound to and occupies part of the ATP-binding site. Disruption of the hydrophobic interaction occurs between the glycine-rich loop and the activation segment (due to phosphorylation in the activation segment).[55,56] The DFG-IN conformation has no allosteric movement and no allosteric binding site. In this conformation, inhibitors or agonists form interactions with the hinge region by forming ∼1−3 hydrogen bonds and the adenine region (ATP binding site) by hydrophobic interaction and also interact with other parts of the ATP binding site.[54]

The αC-helix near the ATP-binding site is called the αC-IN position. In this catalytic position, the Glu501 residue of the αC-helix causes the formation of a salt bridge with the conserved K483 residue. The αC-helix away from the ATP site is referred to as the αC-OUT position.[57,58]

3. CURRENT INSIGHT INTO BRAF INHIBITORS

BRAF binding modes are classified into four basic categories based on the conformation of the binding pocket of the DFG

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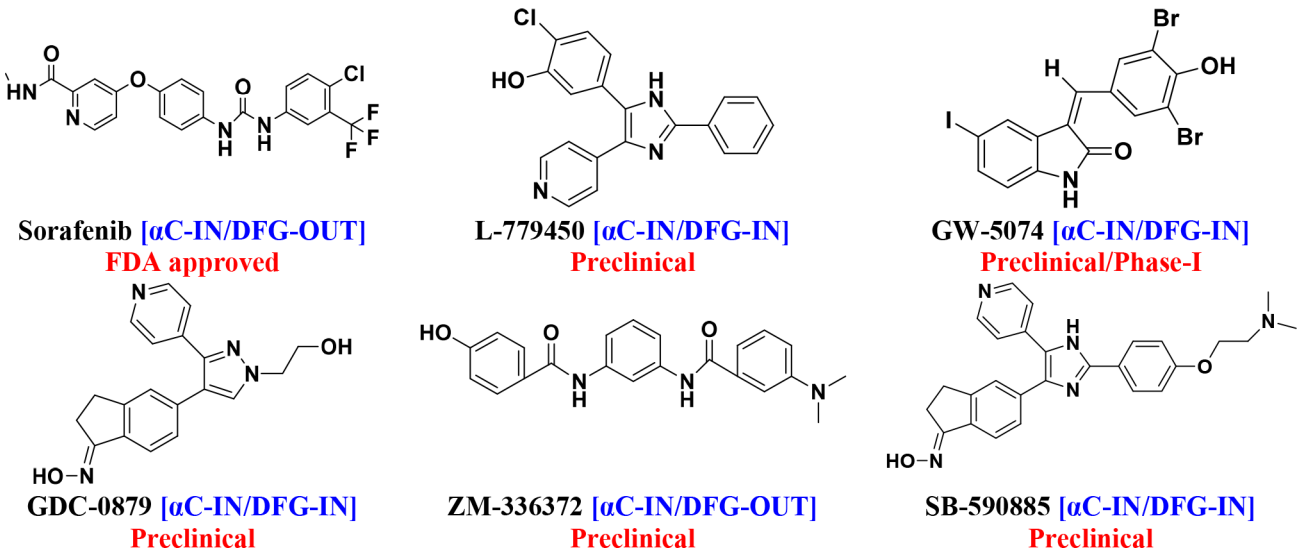

Figure 6. First-generation BRAF inhibitors.

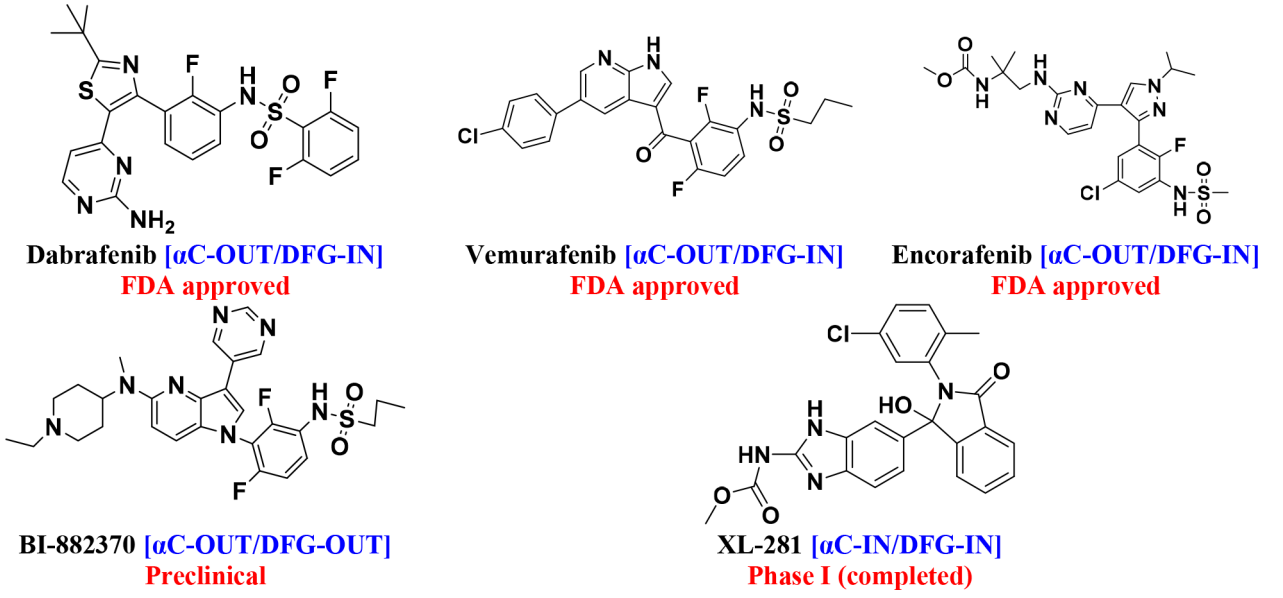

Figure 7. Second-generation BRAF inhibitors.

motif and the αC-helix.[59] These conformations classify inhibitors as follows and are shown in (Figure 5).

1. Type I inhibitors (αC-IN/DFG-IN) **2.** Type II inhibitors (αC-IN/DFG-OUT) **3.** Type $I_{1/2}$ or Type III (αC-OUT/DFG-IN) **4.** $Type_{I/II}$ or Type IV (αC-OUT/DFG-OUT).[60−63]

3.1. First-Generation BRAF Inhibitors: (αC-IN). Originally, this class was designed to target the RAS (CRAF) mutant in the MAPK pathway before the BRAF mutation was discovered.[58,64] The first-generation inhibitors (Figure 6) are designed as ATP-competitive small molecules with αC-IN conformation. They are BRAF monomer inhibitors and bind in dimers to the active site of a protomer within an RAF. They are not selective enough to block mutant RAF dimers.[65,66]

Sorafenib (formally known as BAY43−9006) is a "multi-targeted" RAF kinase inhibitor that inhibits both BRAF and CRAF. It is a nonselective RAF kinase inhibitor. It was the first Food and Drug Administration (FDA)-approved oral drug in 2005, targeting monomeric BRAF tumors (wild-type and V600E mutant) but ineffective against dimeric mutant.[67−71] In addition to sorafenib, many small molecules have been developed as BRAF inhibitors, but most are still in the preclinical phase. L-779,450 is a selective BRAF kinase inhibitor, but it lacks relative therapeutic efficacy due to poor bioavailability.[72−75] GW-5074 (GlaxoSmithKline (GSK)) is an RAF inhibitor with stronger (10-fold) inhibitory activity on RAF1 than on BRAF. A phase I study demonstrated the safety and antitumor efficacy of GW-5074 (MG−005) in combination with sorafenib, providing a unique method of antitumor activity that targets cancer cell necroptosis caused by mitochondrial dysfunction and differing from conventional Raf inhibitor therapies.[76−80] Clinical studies have shown that GDC-0879 is a potent and selective $BRAF^{V600E}$ inhibitor in melanoma and colorectal cancer, inhibiting the RAF/MEK/ERK pathway.[81−83] ZM-336372 is a pan-RAF inhibitor that was the first BRAF inhibitor investigated but simultaneously hyperactivates and inhibits CRAF, resulting in paradoxical CRAF activity without pathway activation.[78,84−86] SB-590885 is a triarylimidazole moiety that has greater potency and

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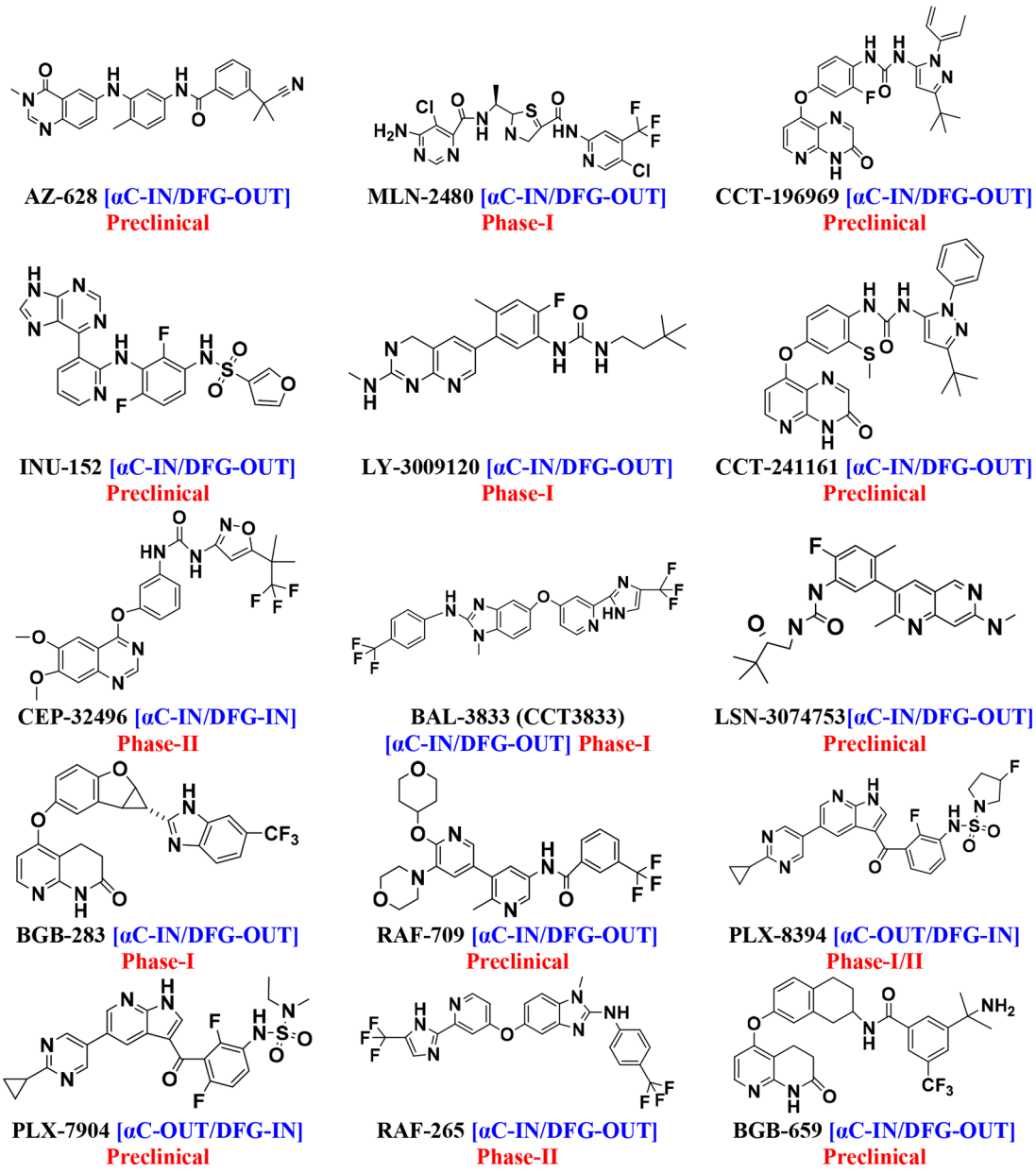

Figure 8. Third-generation BRAF inhibitors.

activity for $BRAF^{V600E}$ mutant than for CRAF in RAF kinases.[87−89]

3.2. Second-Generation BRAF Inhibitors: (αC-OUT). Following the identification of BRAF mutations in 2002, second-generation drugs were found by screening inhibitors for the $BRAF^{V600E48}$ mutant. The second generation (Figure 7) has an αC-out binding conformation with specific selectivity for the tumor driven by the BRAF monomer to target the $BRAF^{V600E}$ mutant with a broad therapeutic window. As monotherapies or in combination with other targeted treatments, these inhibitors significantly improve clinical outcomes for patients with $BRAF^{V600}$ mutation-driven melanoma and some solid malignancies.[53,75,90]

Dabrafenib is an ATP-competitive inhibitor of BRAF kinases was first developed under the name GSK2118436 by GlaxoSmithKline (GSK). It suppresses $BRAF^{V600E}$ more effectively than $BRAF^{V600WT}$. The Biopharmaceutics Categorization System (BCS) classifies dabrafenib as a class II.[91−95] Vemurafenib is the first drug approved by the FDA in 2011 for the treatment of patients with advanced BRAF exon 15 ^{V600E} mutation metastatic melanoma.[96−99] In June 2018, the FDA approved encorafenib (LGX-818), a pyrazolo-pyrimidine-based phenylsulfonamide derivative, for the treatment of $BRAF^{V600E/K}$-mutated cancer. Encorafenib, a selective inhibitor of BRAF kinase, is being paired with binimetinib, an MEK inhibitor, to treat metastatic and advanced malignant melanoma. Compared with other second-generation BRAF inhibitors, encorafenib has a longer duration of action.[100−103] BI882370 inhibited the growth of human BRAF-mutated melanoma cells with 100-fold greater efficacy (1−10 nmol/L) than vemurafenib. BI-882370 is a potent and selective inhibitor of both the $BRAF^{V600E}$ oncogenic mutant and BRAF wild-type.[104−106] XL-281 is an orally bioavailable RAF inhibitor that is generally well-tolerated. XL-281 has a lower rate of keratoacanthoma and squamous cell carcinoma (4%) than

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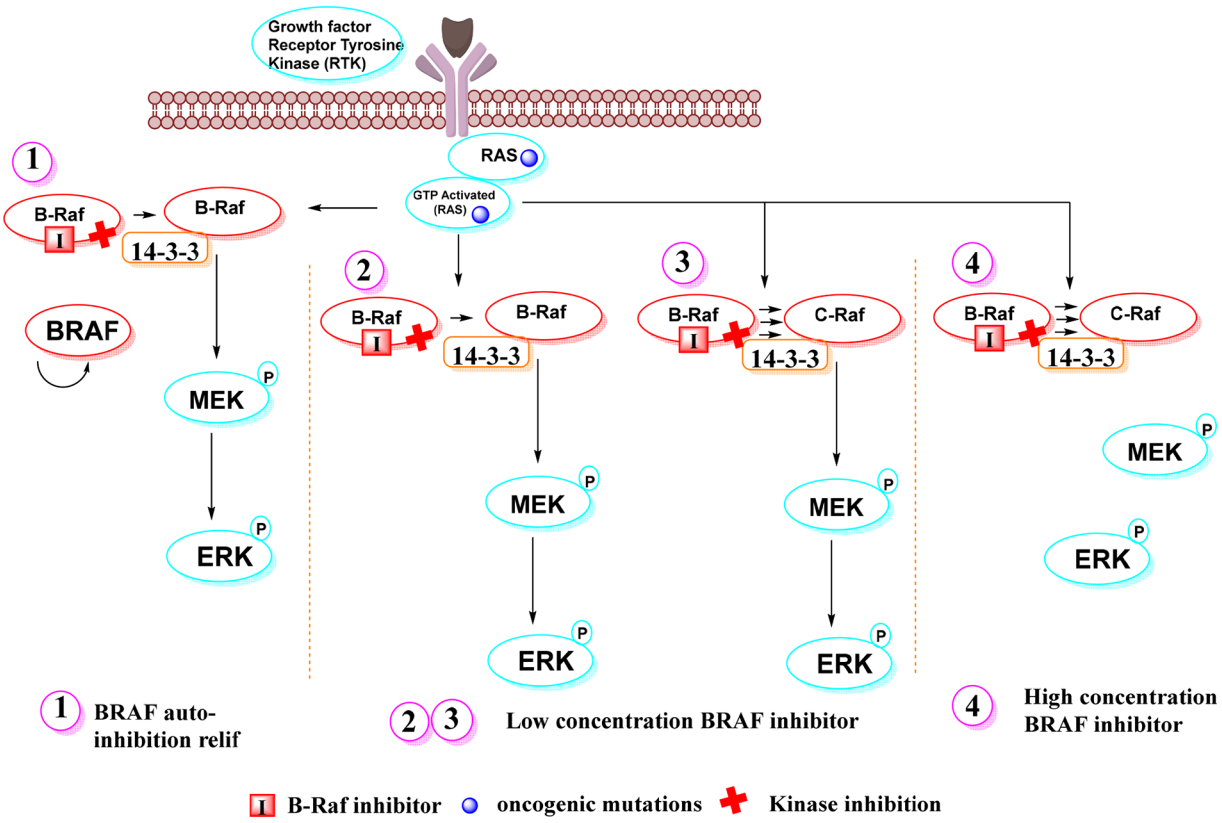

Figure 9. Paradoxical activation. Note: Mechanism of autoinhibition: (1) In this case, inhibition of BRAF in the presence of a mutant or growth factor-activated RAS leads to abrogation of BRAF autoinhibition, so that it homodimerizes with BRAF and becomes hyperactivated. Conformational changes: (2, 3) At low doses, the drug binds only one RAF protomer and causes the other to transactivate. (4) At high doses, it binds to and inhibits both RAF dimers, effectively knocking down the signaling complex.

BRAF inhibitors such as dabrafenib (6−10%) and vemurafenib (18−26%).[107−109]

3.3. Third-Generation BRAF Inhibitors: (αC-IN with Allosteric Binding Site). Third-generation drugs mainly have the αC-IN/DFG-OUT conformation (Figure 8). They were developed to overcome the problems with BRAF dimerization and paradoxical activation. These inhibitors with αC-IN conformation have specific selectivity for tumors driven by BRAF monomers and RAS-dependent BRAF dimers. They have target specificity for BRAF monomers and RAF dimers with a narrow therapeutic window. These inhibitors with αC-OUT conformation have specific selectivity for tumor-driven BRAF monomers and RAS-independent BRAF dimers. They have target specificity for BRAF homodimers and RAF monomers with a broad therapeutic window. The DFG-OUT conformation exposes an additional hydrophobic binding site directly adjacent to the ATP-binding site, commonly referred to as the "allosteric site." Some third-generation drugs are pan-RAF inhibitors that inhibit paradoxical activation and are also not activated during RAF dimerization.[64,66,110,111]

AZ-628 is more effective against the $BRAF^{V600E}$ mutation. AZ-628 compared with sorafenib has shown excellent preclinical results in terms of catalytic inhibition of BRAF protomers and dimers.[112−115] MLN-2480 has a wider cerebral distribution. MLN-2480 is an oral, experimental phase I $BRAF^{V600E}$ inhibitor with a delayed inactivation rate. The allosteric kinase inhibitor MLN-2480 is used to treat melanoma, colorectal, lung, and pancreatic cancer.[116−119] CCT-241161 and CCT-196969 are equally effective against $BRAF^{V600E}$, CRAF, and SFKs (Src family kinase). The development of $BRAF^{V600E}$ skin tumors was inhibited by CCT-241161 and CCT-196969. The efficacy of CCT-241161 and CCT-196969 was observed in PDXs derived from patients exhibiting innate resistance to vemurafenib.[78,120−122] INU-152 only partially activates the paradoxical pathway in melanoma cells with mutated RAS.[120,123] LY-3009120 inhibits the isoforms of ARAF, BRAF, and CRAF with comparable affinity. LY-3009120 stimulates dimerization of BRAF-CRAF and causes paradoxical ERK activation.[120,124−126] CEP-32496 is an orally bioavailable potent BRAF inhibitor. It is also known as agerafenib or RXDX-105. CEP-32492 shows high affinity against $BRAF^{V600E}$. Agerafenib (CEP-32496) inhibited activation of the ERK, MAPK pathway in neuroblastoma cells.[127−131]

BAL−3833 (CCT-3833) works well in in KRAS-mutated tumors because RAF and SRC are important junctions in them. It inhibits both the monomeric and dimeric forms of BRAF.[78,112,132,133] LSN-307453, which inhibits dimerization of all RAF isoforms, is currently in preclinical testing. LSN-3074753 is more active against all ten mutations than $BRAF^{V600E}$.[120,134−136] BGB-283 (lifirafenib) inhibits the BRAF dimer with potent reversible inhibition of all RAFs as well as EGFR.[120,137,138] RAF-709 inhibits monomers and dimers alike.[120,139,140] PLX-8394 inhibits paradoxical formation of RAF dimers and is more potent than vemurafenib. It is a BRAF inhibitor that can be taken orally and does not lead to paradoxical activation of MAPK.[141−144] Compared to vemurafenib, PLX-7904 inhibits MAPK signaling for a longer period of time, resulting in greater blockade of proliferation and lower

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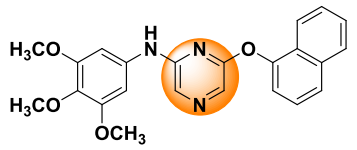

6-(naphthalen-1-yloxy)-N-(3,4,5 trimethoxyphenyl)pyrazin-2-amine

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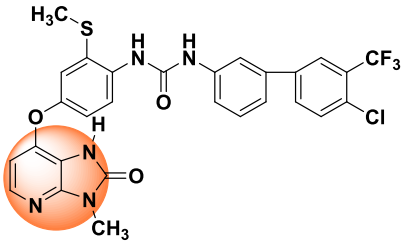
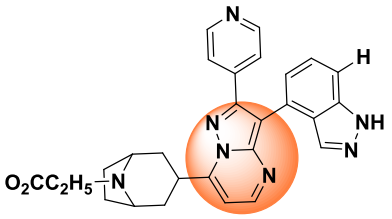


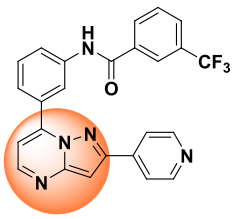
1-(4'-chloro-3'-(trifluoromethyl)-[1,1'-biphenyl]-3-yl)-3-(4-((3-methyl-2-oxo-2,3-dihydro-1H-imidazo[4,5-b]pyridin-7-yl)oxy)-2-(methylthio)phenyl)urea

(Cpd No. 2)



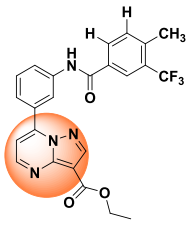
7-(8-ethyl-8-azabicyclo[3.2.1]octan-3-yl)-3-(1H-indazol-4-yl)-2-(pyridin-4-yl)pyrazolo[1,5-a]pyrimidine compound with carbon dioxide (1:1)

(Cpd No. 3)



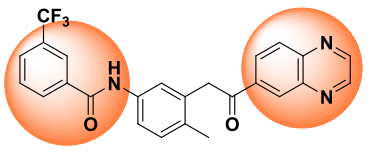
N-(3-(2-(pyridin-4-yl)pyrazolo[1,5-a]pyrimidin-7-yl)phenyl)-3-(trifluoromethyl)benzamide

(Cpd No. 4)



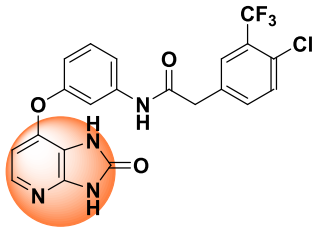
Ethyl 7-(3-(4-methyl-3-(trifluoromethyl)benzamido)phenyl)pyrazolo[1,5-a]pyrimidine-3-carboxylate

(Cpd No. 5)



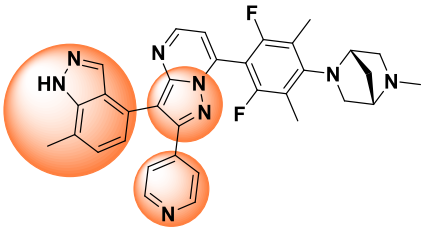
N-(4-methyl-3-(2-oxo-2-(quinoxalin-6-yl)ethyl)phenyl)-3-(trifluoromethyl)benzamide

(Cpd No. 6)



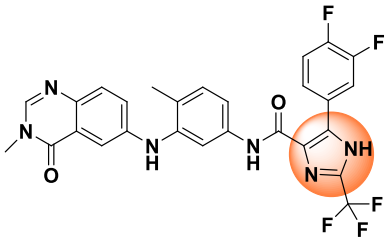
2-(4-chloro-3-(trifluoromethyl)phenyl)-N-(3-((2-oxo-2,3-dihydro-1H-imidazo[4,5-b]pyridin-7-yl)oxy)phenyl)acetamide

(Cpd No. 7)



7-(2,6-difluoro-3,5-dimethyl-4-((1S,4S)-5-methyl-2,5-diazabicyclo[2.2.1]heptan-2-yl)phenyl)-3-(7-methyl-1H-indazol-4-yl)-2-(pyridin-4-yl)pyrazolo[1,5-a]pyrimidine

(Cpd No. 8)



5-(3,4-difluorophenyl)-N-(4-methyl-3-((3-methyl-4-oxo-3,4-dihydroquinazolin-6-yl)amino)phenyl)-2-(trifluoromethyl)-1H-imidazole-4-carboxamide

(Cpd No. 9)

Figure 10. continued

https://doi.org/10.1021/acsomega.3c00332
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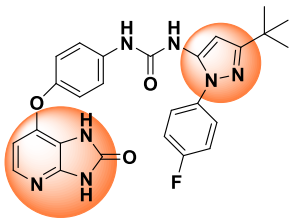
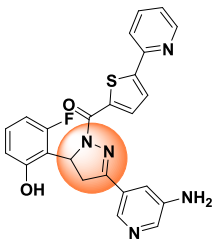


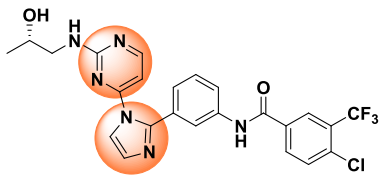
1-(3-(tert-butyl)-1-(4-fluorophenyl)-1H-pyrazol-5-yl)-3-(4-((2-oxo-2,3-dihydro-1H-imidazo[4,5-b]pyridin-7-yl)oxy)phenyl)urea

(Cpd No. 10)



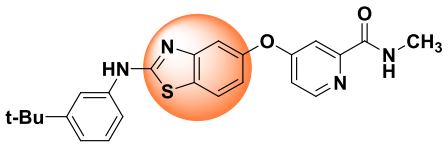
(3-(5-aminopyridin-3-yl)-5-(2-fluoro-6-hydroxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(5-(pyridin-2-yl)thiophen-2-yl)methanone

(Cpd No. 11)



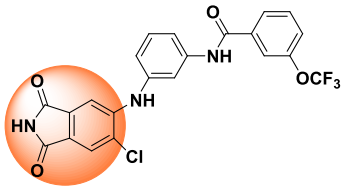
(S)-4-chloro-N-(3-(1-(2-((2-hydroxypropyl)amino)pyrimidin-4-yl)-1H-imidazol-2-yl)phenyl)-3-(trifluoromethyl)benzamide

(Cpd No. 12)



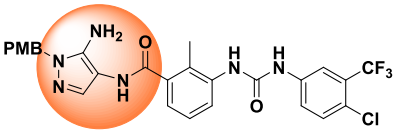
4-((2-((3-(tert-butyl)phenyl)amino)benzo[d]thiazol-5-yl)oxy)-N-methylpicolinamide

(Cpd No. 13)



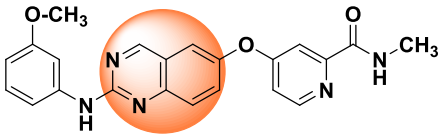
N-(3-((6-chloro-1,3-dioxoisoindolin-5-yl)amino)phenyl)-3-(trifluoromethoxy)benzamide

(Cpd No. 14)



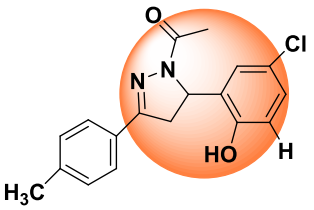
N-(5-amino-1-(4-methoxybenzyl)-1H-pyrazol-4-yl)-3-(3-(4-chloro-3-(trifluoromethyl)phenyl)ureido)-2-methylbenzamide

(Cpd No. 15)



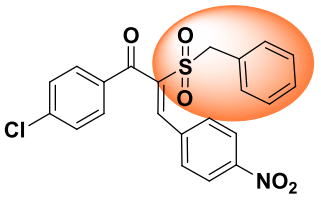
4-((2-((3-methoxyphenyl)amino)quinazolin-6-yl)oxy)-N-methylpicolinamide

(Cpd No. 16)



1-(5-(5-chloro-2-hydroxyphenyl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)ethan-1-one

(Cpd No. 17)



(Z)-2-(benzylsulfonyl)-1-(4-chlorophenyl)-3-(4-nitrophenyl)prop-2-en-1-one

(Cpd No. 18)

Figure 10. continued

https://doi.org/10.1021/acsomega.3c00332
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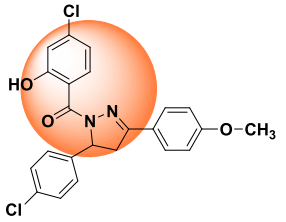
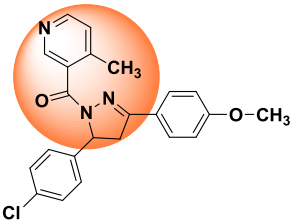


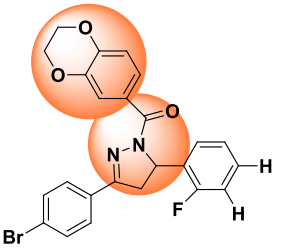
(4-chloro-2-hydroxyphenyl)(5-(4-chlorophenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)methanone

(Cpd No. 19)



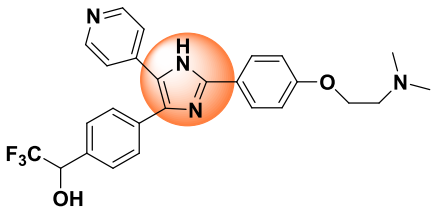
(5-(4-chlorophenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(4-methylpyridin-3-yl)methanone

(Cpd No. 20)



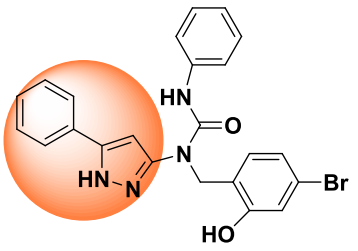
(3-(4-bromophenyl)-5-(2-fluorophenyl)-4,5-dihydro-1H-pyrazol-1-yl)(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)methanone

(Cpd No. 21)



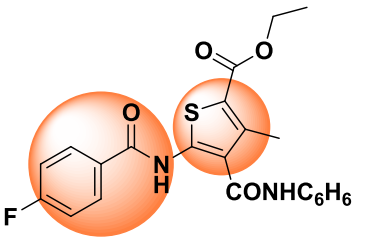
1-(4-(2-(4-(2-(dimethylamino)ethoxy) phenyl)-5-(pyridin-4-yl)-1H-imidazol-4-yl)phenyl)-2,2,2-trifluoroethan-1-ol

(Cpd No. 22)



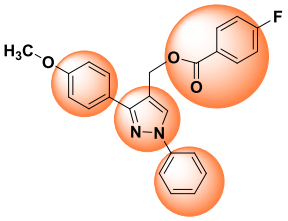
1-(4-bromo-2-hydroxybenzyl)-3-phenyl-1-(5-phenyl-1H-pyrazol-3-yl)urea

(Cpd No. 23)



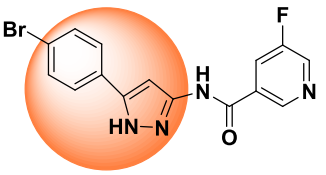
ethyl 5-(4-fluorobenzamido)-4-(hexa-1,3,5-triyn-1-ylcarbamoyl)-3-methylthiophene-2-carboxylate compound with dihydrogen (1:2)

(Cpd No. 24)



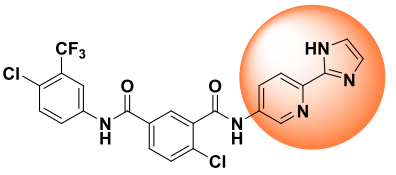
(3-(4-methoxyphenyl)-1-phenyl-1H-pyrazol-4-yl) methyl 4-fluorobenzoate

(Cpd No. 25)



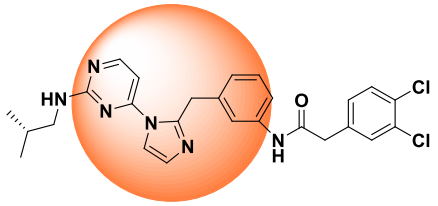
N-(5-(4-bromophenyl)-1H-pyrazol-3-yl)-5-fluoronicotinamide

(Cpd No. 26)



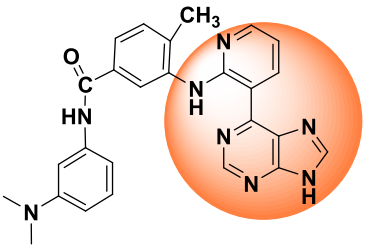
N3-(6-(1H-imidazol-2-yl)pyridin-3-yl)-4-chloro-N1-(4-chloro-3-(trifluoromethyl)phenyl)isophthalamide

(Cpd No. 27)



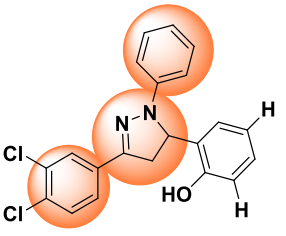
2-(3,4-dichlorophenyl)-N-(3-((1-(2-(isobutylamino)pyrimidin-4-yl)-1H-imidazol-2-yl)methyl)phenyl)acetamide

(Cpd No. 28)



3-((3-(9H-purin-6-yl)pyridin-2-yl)amino)-N-(3-(dimethylamino) phenyl)-4-methylbenzamide

(Cpd No. 29)



2-(3-(3,4-dichlorophenyl)-1-phenyl-4,5-dihydro-1H-pyrazol-5-yl)phenol

(Cpd No. 30)

Figure 10. continued

https://doi.org/10.1021/acsomega.3c00332
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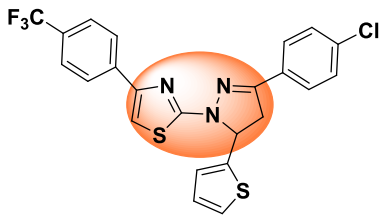
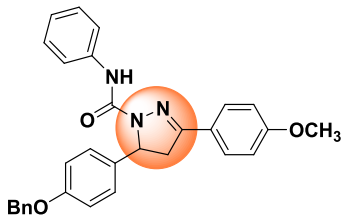


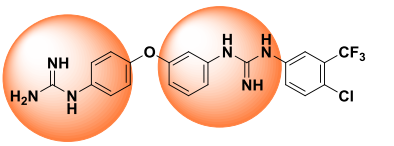
2-(3-(4-chlorophenyl)-5-(thiophen-2-yl)-4,5-dihydro-1H-pyrazol-1-yl)-4-(4-(trifluoromethyl)phenyl) thiazole

(Cpd No. 31)



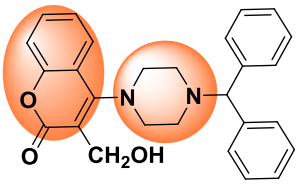
5-(4-(benzyloxy)phenyl)-3-(4-methoxyphenyl)-N-phenyl-4,5-dihydro-1H-pyrazole-1-carboxamide

(Cpd No. 32)



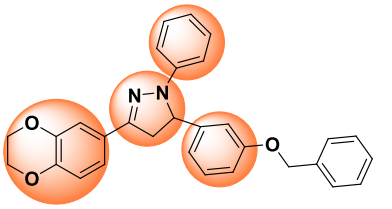
1-(4-chloro-3-(trifluoromethyl)phenyl)-3-(3-(4-guanidino phenoxy) phenyl) guanidine

(Cpd No. 33)



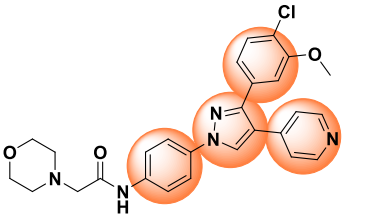
4-(4-benzhydrylpiperazin-1-yl)-3-(hydroxymethyl)-2H-chromen-2-one

(Cpd No. 34)



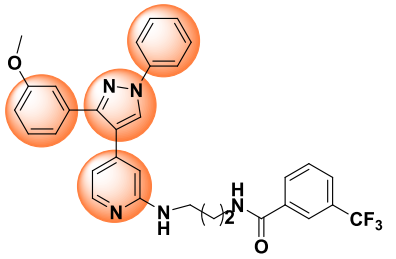
5-(3-(benzyloxy)phenyl)-3-(2,3-dihydrobenzo[b][1,4]dioxin-6-yl)-1-phenyl-4,5-dihydro-1H-pyrazole

(Cpd No. 35)



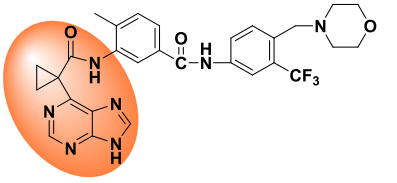
N-(4-(3-(4-chloro-3-methoxyphenyl)-4-(pyridin-4-yl)-1H-pyrazol-1-yl)phenyl)-2-morpholinoacetamide

(Cpd No. 36)



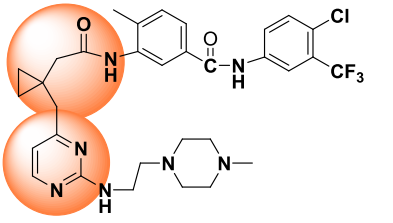
N-(2-((4-(3-(3-methoxyphenyl)-1-phenyl-1H-pyrazol-4-yl)pyridin-2-yl)amino) ethyl)-3-(trifluoromethyl)benzamide

(Cpd No. 37)



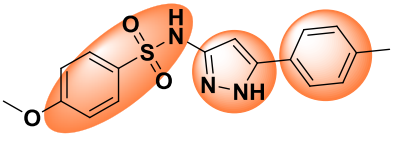
3-(1-(9H-purin-6-yl)cyclopropane-1-carboxamido)-4-methyl-N-(4-(morpholinomethyl)-3-(trifluoromethyl)phenyl)benzamide

(Cpd No. 38)



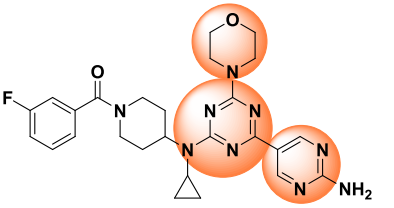
N-(4-chloro-3-(trifluoromethyl)phenyl) -4-methyl-3-(2-(1-((2-((2-(4-methylpiperazin-1-yl)ethyl) amino) pyrimidin-4-yl)methyl) cyclopropyl) acetamido)benzamide

(Cpd No. 39)



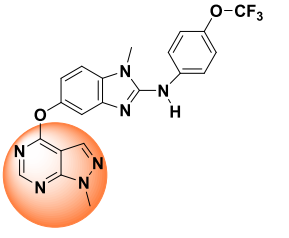
4-methoxy-N-(5-(p-tolyl)-1H-pyrazol-3-yl)benzenesulfonamide

(Cpd No. 40)



(4-((4-(2-aminopyrimidin-5-yl)-6-morpholino-1,3,5-triazin-2-yl)(cyclopropyl)amino)piperidin-1-yl)(3-fluorophenyl)methanone

(Cpd No. 41)



1-methyl-5-((1-methyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)oxy)-N-(4-(trifluoromethoxy)phenyl)-1H-benzo[d]imidazol-2-amine

(Cpd No. 42)

Figure 10. continued

https://doi.org/10.1021/acsomega.3c00332
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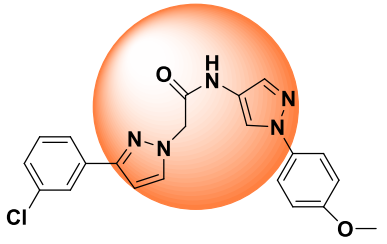
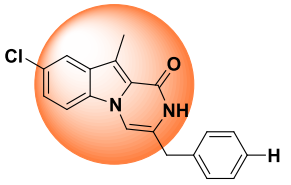


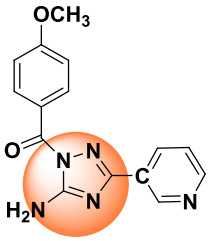
2-(3-(3-chlorophenyl)-1H-pyrazol-1-yl)-N-(1-(4-methoxyphenyl)-1H-pyrazol-4-yl) acetamide

(Cpd No. 43)



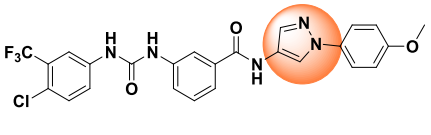
3-benzyl-8-chloro-10-methylpyrazino[1,2-a]indol-1(2H)-one

(Cpd No. 44)



(5-amino-3-(pyridin-3-yl)-1H-1,2,4-triazol-1-yl)(4-methoxyphenyl) methanone

(Cpd No. 45)



3-(3-(4-chloro-3-(trifluoromethyl) phenyl)ureido)-N-(1-(4-methoxyphenyl)-1H-pyrazol-4-yl)benzamide

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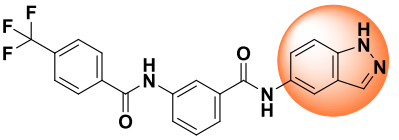
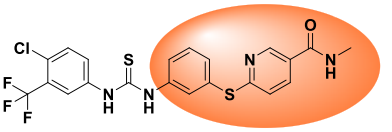


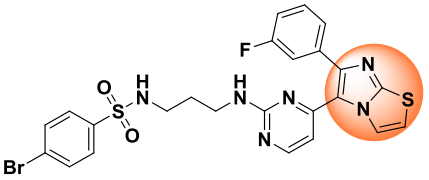
N-(1H-indazol-5-yl)-3-(4-(trifluoromethyl)benzamido)benzamide

(Cpd No. 47)



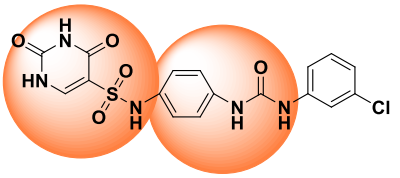
6-((3-(3-(4-chloro-3-(trifluoromethyl) phenyl)thioureido)phenyl)thio)-N-methylnicotinamide

(Cpd No. 48)



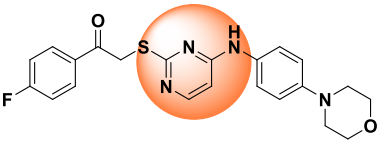
4-bromo-N-(3-((4-(6-(3-fluorophenyl) imidazo[2,1-b]thiazol-5-yl)pyrimidin-2-yl) amino)propyl) benzenesulfonamide

(Cpd No. 49)



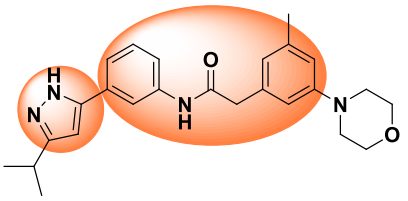
N-(4-(3-(3-chlorophenyl) ureido)phenyl)-2,4-dioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonamide

(Cpd No. 50)



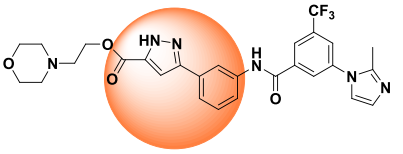
1-(4-fluorophenyl)-2-((4-((4-morpholinophenyl)amino)pyrimidin-2-yl)thio)ethan-1-one

(Cpd No. 51)



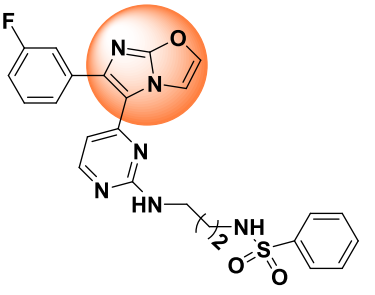
N-(3-(3-isopropyl-1H-pyrazol-5-yl)phenyl)-2-(3-methyl-5-morpholinophenyl)acetamide

(Cpd No. 52)



2-morpholinoethyl 3-(3-(3-(2-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl) benzamido)phenyl)-1H-pyrazole-5-carboxylate

(Cpd No. 53)



N-(2-((4-(6-(3-fluorophenyl) imidazo[2,1-b]oxazol-5-yl)pyrimidin-2-yl)amino) ethyl) benzenesulfonamide

(Cpd No. 54)

Figure 10. continued

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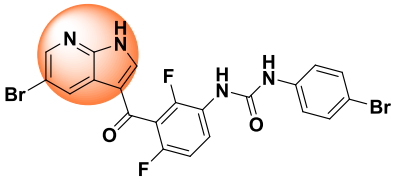
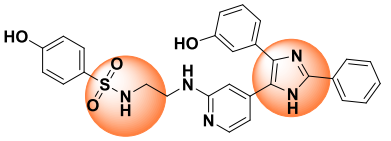


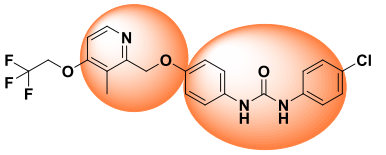
1-(3-(5-bromo-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluorophenyl)-3-(4-bromophenyl)urea

(Cpd No. 55)



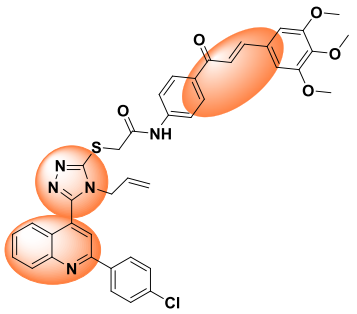
4-hydroxy-N-(2-((4-(4-(3-hydroxyphenyl)-2-phenyl-1H-imidazol-5-yl)pyridin-2-yl)amino)ethyl) benzenesulfonamide

(Cpd No. 56)



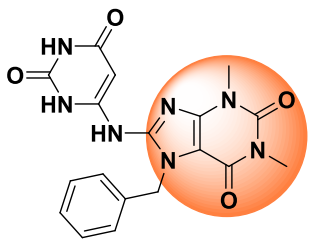
1-(4-chlorophenyl)-3-(4-((3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl)methoxy)phenyl)urea

(Cpd No. 57)



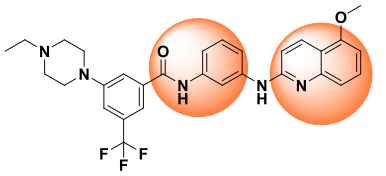
(E)-2-((4-allyl-5-(2-(4-chlorophenyl) quinolin-4-yl)-4H-1,2,4-triazol-3-yl)thio)-N-(4-(3-(3,4,5-trimethoxyphenyl) acryloyl)phenyl)acetamide

(Cpd No. 58)



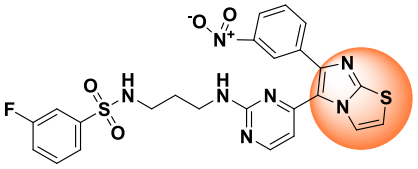
7-benzyl-8-((2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)amino)-1,3-dimethyl-3,7-dihydro-1H-purine-2,6-dione

(Cpd No. 59)



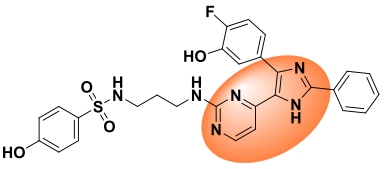
3-(4-ethylpiperazin-1-yl)-N-(3-((5-methoxyquinolin-2-yl)amino)phenyl)-5-(trifluoromethyl)benzamide

(Cpd No. 60)



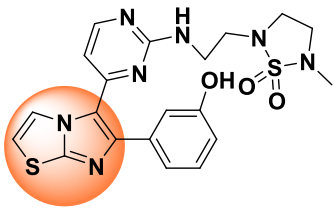
3-fluoro-N-(3-((4-(6-(3-nitrophenyl)imidazo[2,1-b]thiazol-5-yl)pyrimidin-2-yl)amino) propyl)benzenesulfonamide

(Cpd No. 61)



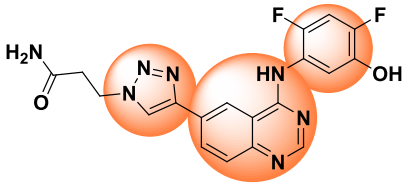
N-(3-((4-(4-(4-fluoro-3-hydroxyphenyl)-2-phenyl-1H-imidazol-5-yl)pyrimidin-2-yl)amino)propyl)-4-hydroxybenzenesulfonamide

(Cpd No. 62)



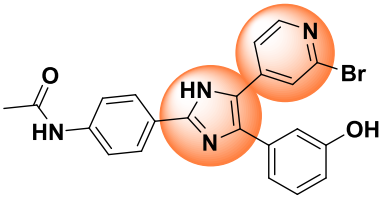
2-(2-((4-(6-(3-hydroxyphenyl) imidazo[2,1-b]thiazol-5-yl)pyrimidin-2-yl)amino)ethyl)-5-methyl-1,2,5-thiadiazolidine 1,1-dioxide

(Cpd No. 63)



3-(4-(4-((2,4-difluoro-5-hydroxyphenyl)amino)quinazolin-6-yl)-1H-1,2,3-triazol-1-yl)propanamide

(Cpd No. 64)



N-(4-(5-(2-bromopyridin-4-yl)-4-(3-hydroxyphenyl)-1H-imidazol-2-yl) phenyl)acetamide

(Cpd No. 65)

Figure 10. Various synthesized compounds with parents' scaffolds as BRAF mutant inhibitors. Note: (*) Cpd No.: Compound number; (**) Parent scaffold: Different derivatives of the parent scaffold were synthesized, but only the most potent compounds based on cell line or enzyme kinase activity were chosen.

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Table 1. Different Scaffolds and Their Key Aspects in BRAF Mutant

Compound No.	Parent scaffold*	$BRAF^{V600E}$(Enzyme Kinase)/Cell line	IC_{50} (μM)	Key points	Refs
1	Pyrazine	$BRAF^{V600E}$	1.2	Pyrazine scaffold shows strong selectivity against $BRAF^{V600E}$ mutant compared with CRAF	155
2	Imidazo-pyridine	$BRAF^{V600E}$	0.001	Demonstrated interaction with the DFG pocket	156
3	Pyrazolo-pyrimidine	WM266−4	0.30	Bound to the active conformation of BRAF. Fusion of the tropane moiety with the pyrimidine core led to novel, potent BRAF inhibitors that unfortunately did not show good efficacy in cells.	157
4	Pyrazolo-pyrimidine	A375	0.28	Introduction of groups interacting with the hinge region of the kinase in the 2-position of the scaffold with potent BRAF inhibitors	158
5	Pyrazolo-pyrimidine	$BRAF^{V600E}$	0.5	Replacement of esters with amides was tolerated with a slight increase in enzyme potency	159
6	Amidoheteroaryls (quinoxaline-benzamide)	$BRAF^{V600E}$	0.001	Association with DFG-out allosteric inhibitors of BRAF kinase	160
7	Imidazo-pyridinone	$BRAF^{V600E}$	0.015	Inhibitors of the type II BRAF, in which the central phenyl ring is linked to the hinge binder and 1,3 substitutions bind to the allosteric pocket of BRAF	161
8	Indazolylpyrazolopyrimidine	A375	0.024	Type I BRAF inhibitors. Induced dimerization/activation mechanism. In BRAF mutant xenograft models, it shows good inin vivo good anticancer activity	162
9	Imidazole	$BRAF^{V600E}$	0.3	Allosteric DFG-OUT inhibitors of BRAF kinase. The quinazoline moiety binds to the hinge region, the phenyl ring binds to the gatekeeper residue, and the imidazole moiety binds to the selectivity site.	163
10	Imidazopyridin -Imidazole	BRAF	0.142	Type II kinase inhibitors with DFG-OUT pocket conformation. Substituted phenyl groups as C-rings (adjacent to imidazole) resulted in a significant improvement in cellular potency	164
11	Pyrazoline	$BRAF^{V600E}$	4.0	Chemical modification of the pyrazoline scaffold led to the development of a potent and selective $BRAF^{V600E}$ inhibitor. (2-Fluoro,6-hydroxy) substitution enhances cellular potency	165
12	Pyrimidin-4-yl-1*H*-imidazole	WM3629	0.62	Superior antiproliferative activities compared to sorafenib. Selective and potent CRAF inhibitor.	166
13	Benzothiazole	$BRAF^{V600E}$	0.004	DFG-OUT pocket conformation in which substituted anilines replace Phe593, which swings out and interacts with aromatic systems in the hinge region and selectivity pocket.	167
14	Isoindoline-1,3-diones	$BRAF^{V600E/WT}$	0.006	DFG-OUT pocket conformation. Structure-based drug design technology was used to develop both type I and type II BRAF inhibitors with unique chemical scaffolds and hinge binders.	168
15	Imidazolopyrazole	A375	0.27	The compound showed competitive antiproliferative activity to sorafenib. Potential and selective $BRAF^{V600E}$ and CRAF inhibitor	169
16	Quinazoline	$BRAF^{V600E}$	0.07	DFG-OUT conformation	170
17	4,5-Dihydro-2*H*-pyrazole 2-hydroxyphenyl	$BRAF^{V600E}$	0.22	Compound showed potent and strong anti-$BRAF^{V600E}$ activity	171
18	Benzylsulfonyl chalcone	$BRAF^{V600E}$	0.17	More potent and selective $BRAF^{V600E}$ inhibitor compared to $BRAF^{WT}$	172
19	Salicylamide moiety containing 3,5-diarylpyrazoline derivatives	$BRAF^{V600E}$	0.16	Selective inhibition of proliferation of $BRAF^{V600E}$ and $BRAF^{WT}$ mutants	173
20	4,5-Dihydro-1*H*-pyrazole niacinamide	$BRAF^{V600E}$	0.20	Compound has highest bioactivity compared to positive control sorafenib.	174
21	2,3-Dihydrobenzo [b][1,4]dioxin-containing 4,5-dihydro-1*H*-pyrazole	$BRAF^{V600E}$	0.11	More potent than erlotinib and compound with selective $BRAF^{V600E}$ inhibitor activity	175
22	Imidazole	$BRAF^{V600E}$	>10	The novel entity benzo[b]thiophen-3-yl)-2,2,2-trifluoroethanol interacts with the BPII pocket of BRAF, resulting in potent BRAF kinase inhibition in vitro and in cells.	176
23	5-Phenyl-1*H*-pyrazol	WM266.4	1.50	Compound showed high antiproliferative activity comparable to positive control vemurafenib	177
24	*N*-(thiophen-2-yl) benzamide	$BRAF^{V600E}$	0.63	Developed compound proves to be a potent $BRAF^{V600E}$ kinase inhibitor	178
25	(1,3-Diphenyl-1H-pyrazol-4-yl) methyl benzoate	WM266.4	0.94	High antiproliferative effect comparable to positive control vemurafenib and highly selective $BRAF^{V600E}$ in vitro inhibitory activity	179
26	5-Phenyl-1*H*-pyrazole	WM266.4	2.63	Compound exhibits highly selective $BRAF^{V600E}$ in vitro inhibitory activity comparable to positive control vemurafenib	180
27	2-(1*H*-Imidazol-2-yl) pyridine	A375	2.93	The nitrogen of the imidazole ring forms a critical hydrogen bond with Cys531 in the hinge region, while the amide moiety forms two additional hydrogen bonds with Glu500 and Asp593 in the DFG region.	181
28	Benzyl 2-(1*H*-imidazole-1-yl) pyrimidine	A375	1.82	Conformation with two different $BRAF^{V600E/V599E}$ mutants and CRAF inhibitor activity	182

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Table 1. continued

Compound No.	Parent scaffold*	$BRAF^{V600E}$(Enzyme Kinase)/Cell line	IC_{50} (μM)	Key points	Refs
29	2-Amino-3-purinylpyridine	A375	1.839	DFG-OUT conformation with $BRAF^{V600E}$ inhibitory activity. The compounds decreased the growth of melanoma cells A375 ($BRAF^{V600E}$) via the ERK pathway, paradoxical activation was not observed in ERK melanoma cells SKMEL-2 ($BRAF^{WT}$)	183
30	Diaryl-pyrazol	$BRAF^{V600E}$	0.15	Addition of ortho-hydroxyl to the 4,5-dihydro-1H-pyrazole scaffold enhanced antitumor activity, while expansion of the group at N-1 of pyrazoline was similarly beneficial. The compound is more potent than vemurafenib and erlotinib.	184
31	4,5-Dihydro-1*H*-pyrazole thiazole	WM266.4	0.12	The entire $BRAF^{V600E}$ complex was characterized as a receptor, and the sphere was selected based on the ligand binding position of SB−590885.	185
32	Pyrazole	$BRAF^{V600E}$	0.63	Potent inhibitory activities against $BRAF^{V600E}$ and $BRAF^{WT}$	186
33	Guanidinium-diaryl	T-29	0.9	DFG-OUT conformations. Sorafenib binds to the adenine region of the ATP-binding site while penetrating deeper into the hydrophobic pocket.	187
34	Benzo-α-pyrone containing piperazine	$BRAF^{V600E}$	0.37	The drug induces cell apoptosis and marked DNA fragmentation. Cell cycle arrest in G0/G1 phase in melanoma cells. Compound tightly binds to the crystal structure of $BRAF^{V600E}$ at the active site.	188
35	Dioxin-containing triaryl pyrazoline	$BRAF^{V600E}$	0.04	Selective for $BRAF^{V600E}$ against $BRAF^{WT}$, CRAF and EGFR and low in toxicity	189
36	1,3,4-Triaryl pyrazole	A375	1.82	The compound was tested against $BRAF^{V600E}$, $BRAF^{WT}$, and C-RAF. Its type 2-kinase inhibitors bind to the inactive form of kinases (DFG-OUT).	190
37	1,3,4-Triaryl pyrazole	SK-MEL-5	1.54	The compound was tested against seven kinases: $BRAF^{WT}$, $BRAF^{V600E}$, RAF1, EGFR, P38a/MAPK14, ABL1, and ABL1 (T315l). Good inhibitory activity against mutant $BRAF^{V600E}$ and RAF1 kinases	191
38	Purine based cyclopropyl formamide fragment	$BRAF^{V600E}$	3.49	Inhibits proliferation of SK-MEL-2 cell lines without paradoxical activation of ERK. DFG-OUT conformation inhibits all subtypes of Raf proteins. More potent than vemurafenib and sorafenib	192
39	Pyrimidine based cyclopropyl formamide fragment	$BRAF^{V600E}$	12.6	The compound was tested against seven kinases: $BRAF^{WT}$, $BRAF^{V600E}$, A-RAF, C-RAF. DFG-OUT, BRAFV600E inhibitors inhibited A375 cell line proliferation via the ERK pathway without paradoxical activation of ERK in SK	193
40	N-(5-Phenyl-1H-pyrazol-3-yl) benzenesulfonamide	WM266.4	1.58	The binding mode of the developed compound is similar to that of the $BRAF^{V600E}$ inhibitor. It consists of a terminal aromatic group (phenyl or pyrazole) filling the allosteric pocket formed by the displacement of the DFG loop and an amide linker connecting an aryl group in the hydrophobic pocket to a hinge-binding heterocycle. The use of an amide linker would allow rapid and efficient screening of many possible hinge-binding groups.	194
41	2-(Aminopyrimidin-5-yl)-4-(morpholin-4-yl)-6-substituted triazine	A375	0.12	Compound was tested against PI3K(α,β,γ), mTOR $BRAF^{V600E}$ $BRAF^{WT}$ and CRAF kinase activity, proving to be a PI3Kα and $BRAF^{V600E}$ dual inhibitor.	195
42	Pyrazolo[3,4-d]pyrimidine	A375	1.74	DFG-OUT-type pan-RAF inhibitors. High inhibitory activities against both $BRAF^{V600E}$ and VEGFR-2 kinase, comparable to sorafenib act as dual $BRAF^{V600E}$ and VEGFR-2 inhibitor	196
43	Pyrazole	A375	0.96	Compound has potential as a $BRAF^{V600E}$ inhibitor compared to vemurafenib	197
44	Pyrazino[1,2-a]indole	A-549	0.8	Compound showed both $BRAF^{V600E}$ and antioxidant activity.	198
45	1,2,4-triazole	H-460	1.6	Compound showed potent $BRAF^{V6OOE}$, EGFR and tubulin inhibitory activity	199
46	Pyrazole	A375	0.39	Type II $BRAF^{V600E}$ inhibitors in this hit compound showed hydrogen bonding with Cys532 in the hinge region, Asp594 in the DFG motif and Glu501 in the αC-helix. The compound showed no significant effect on phosphorylation of ERK and MEK and is more potent than vemurafenib	200
47	Indazole	A375	1.97	The compound does not induce paradoxical effect in normal cells. Shows good inhibitory $BRAF^{V600E}$ activities and antiproliferation activities.	201
48	Thioether and nicotinamide containing sorafenib analogs	B16BL6	19.75	DFG-OUT conformation: the synthesized compound hasBRAF, $BRAF^{V600E}$ and VEGFR-2-muti kinase inhibitor	202
49	Imidazo[2,1-b]thiazole	$BRAF^{V600E}$	9.30	The developed compound was tested against leukemia, nonsmall cell lung cancer, colon, melanoma, ovarian, kidney, prostate, and breast cancer and obtained moderate results. The compound showed the greatest potential inhibitory effect against $BRAF^{V600E}$ compared to $BRAF^{WT}$ and CRAF.	203
50	*N*-phenyl-(2,4-dihydroxypyrimidine-5-sulfonamido) phenylurea	A549	0.67	The compound outperformed BRAF kinase inhibition and EGFR kinase inhibition	44
51	4-Amino-2-thiopyrimidines	$BRAF^{WT}$	0.11	Dual VEGFR-2 and BRAF inhibitors. In the designed compound, the 4-amino-2-thiopyrimidine core scaffold is to be located in the central gate region of the inactive DFG-OUT conformations of both enzymes. The hydrophobic substituent at the 4-amino group occupies the hydrophobic posterior pocket on one side. The substituent on the sulfide group, on the other hand, expands to fit into the hinge region.	204
52	*N*-(3-(3-Alkyl-1*H*-pyrazol-5-yl) phenyl)-aryl amide	$BRAF^{V600E}$	0.70	Superior selectivity over $BRAF^{V600E}$ and CRAF over $BRAF^{WT}$ kinases	205
53	3-Carbonyl-5-phenyl-1*H*-pyrazole	$BRAF^{V600E}$	0.10	Paradoxical activation can be avoided by inhibiting $BRAF^{V600E}$ and CRAF more selectively than $BRAF^{WT}$. In silico studies have shown that 3-carbonyl-5-phenyl-1*H*-pyrazole (3) has three hydrogen bonds near the hinge site. In addition, the pyrazole and middle phenyl rings of the compound interlock with Phe595 of the DFG motif.	206

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Table 1. continued

Compound No.	Parent scaffold*	$BRAF^{V600E}$(Enzyme Kinase)/Cell line	IC_{50} (μM)	Key points	Refs
54	Imidazo[2,1-b]oxazole	$BRAF^{V600E}$	0.034	The developed compound was tested against leukemia, nonsmall cell lung cancer, CNS, colon, melanoma, ovarian, renal, prostate, and breast cancers and obtained moderate results. Most effective against $BRAF^{V600E}$ compared to $BRAF^{WT}$ and RAf1	207
55	Pyrrolo[2,3-b]pyridine	$BRAF^{V600E}$	0.080	Compound with potent $BRAF^{V600E}$ inhibitor	208
56	Imidazole-sulphonamides	$BRAF^{V600E}$	32.90	The developed compound was tested against leukemia, nonsmall cell lung cancer, CNS, colon, melanoma, ovarian, kidney, prostate and breast cancer, achieving moderate results. Compound with potent $BRAF^{V600E}$ inhibitor with active binding site	209
57	1-Aryl-3-[4-(pyridin-2-ylmethoxy)phenyl]urea	A549	2.39	Using the colorimetric MTT technique, compounds were tested in vitro for their antiproliferative activity against cancer cell lines A549, HCT-116, PC−3, and HL7702, a normal human liver cell line. Compound more effective than sorafenib	210
58	Quinoline/chalcone/1,2,4-triazole	$BRAF^{V600E}$	1.1	The compound was tested against pancreatic (Panc-1), breast (MCF−7), colon (HT−29), and epithelial cell lines (A-549). The compound showed binding affinity to the (ATP) active site of $BRAF^{V600E}$ and was comparable to that of vemurafenib. They have potent inhibitory effects on $BRAF^{V600E}$ and EGFR.	211
59	7,8-Disubstituted-1,3-dimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione	$BRAF^{WT}$	115.1	Molecular docking in the active site of $BRAF^{WT}$ revealed that C-8 and C-7 substitutions for the N-7 benzyl and tricyclic derivatives, respectively, were responsible for binding interactions with the DFG loop amino acid Asp593 and the αC-helical amino acid Glu500. The compounds showed promising multikinase activity against PI3Kα, $BRAF^{V600E}$ and $BRAF^{WT}$	212
60	2-Anilinoquinoline-based arylamides	$BRAF^{V600E}$	0.888	The developed compound was tested against leukemia, nonsmall cell lung cancer, CNS, colon, melanoma, ovarian, renal, prostate and breast cancer and obtained moderate results. Inhibitory activity against $BRAF^{WT}$, $BRAF^{V600E}$ (DFG-OUT) and C-RAF kinases	213
61	Imidazothiazole	$BRAF^{V600E}$	0.021	The developed compound was tested against leukemia, nonsmall cell lung cancer, CNS, colon, melanoma, ovarian, kidney, prostate and breast cancer and achieved moderate results Compound with $BRAF^{V600E}$ and $BRAF^{WT}$ inhibitory activity.	214
62	Imidazol-5-yl-pyrimidine	LOX-IMVI	13.90	Association with interactions with RAS, ribose, hydrophobic pockets and hinge region, additional binding with allosteric pocket of BRAF kinase. With $BRAF^{V600E}$/p38α inhibitors.	215
63	Imidazo[2,1-b]thiazole	$BRAF^{V600E}$	0.07	Activity against mutant BRAF was higher than for CRAF and $BRAF^{WT}$. The compounds are more potent than vemurafenib in terms of inhibition of MEK and ERK phosphorylation.	216
64	4-(3-Hydroxy anilino)-6-(1*H*-1,2,3-triazol-4-yl)quinazolines	$BRAF^{V600E}$	0.051	The compound was tested against $BRAF^{V600E}$, CRAF EGFR, $EGFR^{T790M}$, VEGFR-2, and PDGFR-β. It interacts with the DFG-OUT conformation with and strongly inhibits $BRAF^{V600E}$ at low dose compared to CRAF, EGFRT790 M and VEGFR-2.	217
65	Imidazol-5-yl-pyridine	$BRAF^{V600E}$	0.530	The designed compound has been tested against leukemia, nonsmall cell lung cancer, CNS, colon, melanoma, ovarian, renal, prostate and breast cancer and moderate results were obtained. Dual $BRAF^{V600E}$/p38α inhibitors with high affinity in kinase pockets.	218

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viability. In $BRAF^{V600E}$ melanoma cells, PLX-7904 was found to effectively suppress RAF signaling, whereas it had no paradoxical effects on wild-type cells.[120,145−147] RAF-265 is a dual inhibitor of BRAF and VEGFR2, preventing both osteoclast development and resorption. RAF-265 shows synergistic antitumor activity with ZSTK-474 in medullary thyroid cancer.[117,147−149] BGB-659 is able to inhibit class I and class II BRAF mutations because it can bind both monomeric BRAFs and both protomers of an RAF dimer. BGB659 showed higher activity against $BRAF^{WT}$ kinase.[117,136,150,151]

3.4. Challenges with BRAF Inhibitors. *3.4.1. Challenges with First- and Second-Generation BRAF Inhibitors.* First-generation BRAF inhibitors (αC-IN) have a lack of selectivity. They are ineffective against malignancies caused by II and III BRAF mutants due to homo/heterodimerization, leading to a paradoxical outcome.[66,152]

Second-generation αC-OUT BRAF inhibitors are extremely selective for cancers that rely on monomeric BRAF species, resulting in a broad treatment window. Resistance to first- and second-generation BRAF kinase inhibitors is mediated by BRAF dimerization. However, they have negative allostery and paradoxical activation and are ineffective in cancers based on dimer BRAF mutations. Second-generation BRAF inhibitors (αC-OUT/DFG-IN) exhibit paradoxical activation and allosteric transactivation of BRAF dimers. As a result of treatment with these inhibitors, healthy ($BRAF^{WT}$) cells may be stimulated, leading to secondary malignancies. These drugs are highly effective in $BRAF^{V600E}$ tumors; however, non-V600 mutated malignancies are resistant.[153]

Secondary skin lesions such as hyperkeratosis, keratoacanthomas, and squamous cell carcinomas may occur in patients on BRAF inhibitors. Secondary melanomas, gastric and colonic polyps, and recurrences of previous cancers have also been observed in patients on BRAF inhibitors.[154] Combinations of BRAF inhibitors and MEK inhibitors increase response rates and prolong progression-free survival (PFS) and overall survival (OS) in patients with $BRAF^{V600}$ mutated metastatic melanoma. Although long-term effects have been documented, many patients develop acquired resistance to these drugs.[153]

3.4.2. Challenges with Third Generation BRAF Inhibitors. The ability of inhibitors to efficiently and persistently target both protomers is critical for effective inhibition of such dimers. The goal is to develop third-generation BRAF inhibitors that stabilize BRAF in the αC-IN conformation that can bind to both protomers in dimeric structures while inhibiting cellular CRAF inhibits both monomeric and dimeric forms of RAFs or does not allow dimer formation. The ability of both αC-OUT and αC-IN inhibitors to promote transactivating dimerization of RAFs, as well as their general mechanism of inhibition, results in a paradoxical stimulation of ERK signaling. αC-IN inhibitors are more potent than αC-OUT inhibitors in suppressing dimeric RAF activity. αC-OUT and αC-IN inhibitors both stimulate dimerization; however, αC-IN inhibitors often do so more strongly because they greatly increase RAF binding to the active RAS. αC-IN inhibitors promote paradoxical activation more strongly than αC-OUT inhibitors.[39,66]

Paradoxical Activation by BRAF Inhibitors. Oncogenic BRAF dimers show resistance to BRAF inhibitors and cause paradoxical activation. Paradoxical activation in dimerizing cells by BRAF inhibitors can be explained by two different mechanistic explanations (Figure 9). The first hypothesis states that BRAF is autoinhibited. Another proposed mechanism involves BRAF and CRAF conformational changes induced by physical binding of the RAF inhibitor that promote dimer formation between an uninhibited CRAF protomer and BRAF or CRAF bound to the inhibitor. The phenomenon of paradoxical activation is mostly unknown and hypothetical.[49,154]

4. RECENT ADVANCEMENTS

Following the discovery of BRAF mutations in 2002 and their importance in various cancers, many academic scientists/researchers started to work on it to solve the problem related to different BRAF mutations, dimerization, and paradoxical activation. In this context, various scaffold derivatives, such as pyrazine, imidazole, pyridine, pyrazole, pyrimidine, quinoxaline, etc., and their hybrids were synthesized. Their inhibitory activity against various cell lines such as A375, WM266−4, B16BL6, LOX-IMVI, SK-MEL-5 (melanoma), T-29RKO (colorectal wild type), A549 (lung adenocarcinoma), cancer cell lines, etc., and enzyme kinase assay $BRAF^{V600E}$ and $BRAF^{WT}$ were investigated, and their results were published. A summary of the various synthesized scaffolds and their BRAF inhibitory activity and binding conformation in relation to the existing difficulties (from the discovery of BRAF mutant in 2002 to 2022) is reported in Figure 10 and Table 1.

5. CONCLUSION

Currently, three types of BRAF mutations are reported, i.e., class I: monomeric mutants ($BRAF^{V600}$); class II: BRAF homodimer mutants (non-V600); and class III: non-V600 BRAF heterodimers. Currently, FDA-approved targeted therapy specifically targets $BRAF^{V600E}$ mutant monomers but is insufficiently effective against non-V600E dimers. Third-generation pan-RAF inhibitors are particularly intriguing because they were designed as "paradox breakers" that do not cause paradoxical BRAF activation but, rather, perform activation in a specific manner that leads to the development of secondary cancers. The mobility of the DFG motif is critical for the selectivity of BRAF inhibitors. Many inhibitors are designed based on the αC-helix and DFG conformation; they are ineffective for current problems such as dimerization and paradoxical activation. Therefore, a structure-based design is strongly recommended to design BRAF inhibitors that exploit the properties of BRAF binding sites, conformational changes, and DFG rearrangement.

In recent years, researchers have synthesized various BRAF inhibitors, and after compiling the data, among these inhibitors, a few target the second and third classes of BRAF mutations as well as have problems like dimerization and paradoxical activation. They should design inhibitors that have binding affinity in the core active site [the nucleotide binding site (ADP or ATP), the DFG motif, the phospho-acceptor site (activation segment) adjacent to the DFG motif, and the helix] and an allosteric binding pocket. In cases of BRAF dimerization, new molecules should aim to bind to both protomers instead of one. These efforts could reduce the number of paradoxical breakers, which would provide new hope for a fourth-generation drug to overcome the current challenges of BRAF.

6. FUTURE PERSPECTIVE

Many questions regarding the BRAF protein and its regulation remain unanswered. The structure of BRAF kinase is very

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flexible; different ligands can induce different states of the kinase. The differences in structures may explain the selectivity of BRAF inhibitors for active and inactive BRAF. BRAF monomer, homodimer, and heterodimer protein structures have different mutations. Therefore, based on the above-mentioned mutations and the change of their conformations from active to inactive, further studies are needed.

Because of paradoxical activation, alternative methods of inhibiting Raf (BRAF) signaling using ATP-competitive inhibitors are needed. Little is known about the regulatory mechanisms controlling the formation of BRAF homodimers to heterodimers. In the long term, a better knowledge of RAF regulation would help to develop more effective and better tolerated therapies for BRAF-related malignancies.

AUTHOR INFORMATION

Corresponding Author

Pradeep Kumar − *Department of Pharmaceutical Sciences and Natural Products, Central University of Punjab, Bathinda 151401, India;* orcid.org/0000-0002-4284-2826; Email: pradeepyadav27@gmail.com, pradeep.kumar@cup.edu.in

Authors

Ankit Kumar Singh − *Department of Pharmaceutical Sciences and Natural Products, Central University of Punjab, Bathinda 151401, India*

Pankaj Sonawane − *Department of Pharmaceutical Sciences and Natural Products, Central University of Punjab, Bathinda 151401, India*

Adarsh Kumar − *Department of Pharmaceutical Sciences and Natural Products, Central University of Punjab, Bathinda 151401, India*

Harshwardhan Singh − *Department of Pharmaceutical Sciences and Natural Products, Central University of Punjab, Bathinda 151401, India*

Vladislav Naumovich − *Laboratory of Computational Modeling of Drugs, Higher Medical and Biological School, South Ural State University, Chelyabinsk 454008, Russia*

Prateek Pathak − *Laboratory of Computational Modeling of Drugs, Higher Medical and Biological School, South Ural State University, Chelyabinsk 454008, Russia*

Maria Grishina − *Laboratory of Computational Modeling of Drugs, Higher Medical and Biological School, South Ural State University, Chelyabinsk 454008, Russia;* orcid.org/0000-0002-2573-4831

Habibullah Khalilullah − *Department of Pharmaceutical Chemistry and Pharmacognosy, Unaizah College of Pharmacy, Qassim University, Unayzah 51911, Saudi Arabia*

Mariusz Jaremko − *Smart-Health Initiative and Red Sea Research Center, Division of Biological and Environmental Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal 23955-6900, Saudi Arabia*

Abdul-Hamid Emwas − *Core Laboratories, King Abdullah University of Science and Technology, Thuwal 23955-6900, Saudi Arabia*

Amita Verma − *Bioorganic and Medicinal Chemistry Research Laboratory, Department of Pharmaceutical Sciences, Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj 211007, India;* orcid.org/0000-0001-6653-7335

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c00332

Author Contributions

Conceptualization: Pradeep Kumar; Data collection: Adarsh Kumar, Harshwardhan Singh; Writing the manuscript: Ankit Kumar Singh and Pankaj Sonawane; Sketching of figures and data interpretation: Vladislav Naumovich, Prateek Pathak; Writing, review, and final editing of the manuscript: Habibullah Khalilullah, Mariusz Jaremko, Abdul-Hamid Emwas, Amita Verma, Maria Grishina, and Pradeep Kumar.

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Notes

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BRAF	v-RAF murine sarcoma viral homologue B1
CR	Conserved regions
CRD	Cysteine-rich domain
DIF	Dimerization interface
EGFR	Epidermal growth factor receptors
ERK	Extracellular-signal regulated kinase
FDA	Food and drug administration
MAPK	Mitogen-activated protein kinase
MEK	Mitogen extracellular Kinase
RAF	Rapidly Accelerated Fibrosarcoma
RAS	Rat sarcoma
VEGFR-2	Vascular endothelial growth factor receptor-2

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