Acta Pharmaceutica Sinica B 2024;14(5):1895−1923

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

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REVIEW

Targeting RAF dimers in RAS mutant tumors: From biology to clinic

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Received 9 October 2023; received in revised form 2 January 2024; accepted 20 February 2024

KEY WORDS

RAS mutations;
RAF dimerization;
RAF inhibitors;
Cancer therapy;
Drug resistance

Abstract RAS mutations occur in approximately 30% of tumors worldwide and have a poor prognosis due to limited therapies. Covalent targeting of KRAS G12C has achieved significant success in recent years, but there is still a lack of efficient therapeutic approaches for tumors with non-G12C KRAS mutations. A highly promising approach is to target the MAPK pathway downstream of RAS, with a particular focus on RAF kinases. First-generation RAF inhibitors have been authorized to treat BRAF mutant tumors for over a decade. However, their use in RAS-mutated tumors is not recommended due to the paradoxical ERK activation mainly caused by RAF dimerization. To address the issue of RAF dimerization, type II RAF inhibitors have emerged as leading candidates. Recent clinical studies have shown the initial effectiveness of these agents against RAS mutant tumors. Promisingly, type II RAF inhibitors in combination with MEK or ERK inhibitors have demonstrated impressive efficacy in RAS mutant tumors. This review aims to clarify the importance of RAF dimerization in cellular signaling and resistance to treatment in tumors with RAS mutations, as well as recent progress in therapeutic approaches to address the problem of RAF dimerization in RAS mutant tumors.

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Peer review under the responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

https://doi.org/10.1016/j.apsb.2024.02.018

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1. Introduction

Approximately 30% of human tumors harbor RAS mutations, among the most common oncogenic changes[1]. Individuals diagnosed with tumors that carry RAS mutations face a higher likelihood of metastasis and typically experience an unfavorable prognosis[2]. Nonetheless, the development of precise treatments for tumors with RAS mutations is urgently needed. Among the RAS GTPase family members, KRAS has considerably greater oncogenic potential than NRAS and HRAS[3]. Therefore, particular focus should be placed on managing tumors with KRAS mutations. KRAS mutations are the most common and are associated with approximately 90% of pancreatic cancers, 50% of colorectal cancers (CRCs), and 25% of lung cancers[4]. However, KRAS mutations exhibit significantly reduced sensitivity to numerous targeted inhibitors compared to patients with NRAS and HRAS mutations[5]. Reduced sensitivity, along with a high occurrence of drug resistance, leads to low rates of response and short durations of treatment effectiveness in individuals with KRAS mutant tumors. Nevertheless, the KRAS proteins' distinctive molecular structure and extensive biological impact make it difficult to be targeted directly. Solid tumors commonly exhibit KRAS mutations, with G12D, G12V, and G13D being the most prevalent. Less frequent mutations, such as G12C, G12A, and G12R[6], make up less than 10% of cases. Several inhibitors are being developed that directly target mutant KRAS proteins[4], but various oncogenic mutant KRAS proteins function differently[7]. Only KRAS G12C inhibitors (KRAS G12Ci) are therapeutically beneficial and have received clinical approval for use in treating individuals with advanced non-small cell lung cancer (NSCLC)[8,9], while effective medications are still lacking for non-G12C KRAS mutant cancers. Hence, it is crucial and challenging to investigate specific treatment approaches for RAS mutant tumors, specifically those with non-G12C KRAS mutations.

KRAS mutations regulate the growth and survival of cancer cells mainly by triggering the RAF-MEK-ERK (MAPK) signaling pathway, which has been heavily studied to develop drugs[10,11]. Multiple membrane receptors utilize the MAPK pathway as a central hub for transmitting growth signals[12]. Following external stimulation from growth factors, receptor tyrosine kinases (RTKs) initiate the activation of RAS-GTPases, subsequently attracting RAF kinases to the cellular membrane. This event stimulates the formation of homo or heterodimers of RAF, resulting in autophosphorylation of RAF, followed by the activation of MEK and ERK *via* a phosphorylation cascade[13]. ERK then relocates to the cell nucleus and regulates the activation of numerous transcription factors. Sustained activation of MAPK signaling, facilitated by RAS mutations, drives tumorigenesis. Most MAPK pathway inhibitors could cause the reactivation of ERK signaling when resistance develops[10,14]. Thus, effective treatment of RAS mutant tumors relies on achieving robust and enduring suppression of ERK signaling[15,16].

Investigations into essential kinases downstream of KRAS have attracted significant attention in academic research. In particular, there is an increasing fascination with the advancement of medications targeting distinct RAF isoforms or genetic variations. Targeting RAF upstream of the RAF-MEK-ERK cascade is a promising strategy, as the signal is amplified stepwise from RAF to ERK. Nevertheless, the simultaneous elimination of MEK1/2 or ERK1/2 leads to fatality in adult mice, whereas the concurrent elimination of BRAF and CRAF is well tolerated[17]. Additionally, clinical evidence suggests that targeting BRAF V600 has greater efficacy than targeting MEK in patients with BRAF V600 mutant melanoma[18]. Notably, these RAF monomer inhibitors can induce ERK activation in RAS mutant tumor cells[19], making them inappropriate for treating RAS mutant tumors. ERK signaling reactivation relies heavily on the homo and heterodimerization of RAF[20,21]. Preclinical and clinical studies of next-generation RAF dimer inhibitors are ongoing and have obtained promising results, although some drugs still exhibit minimal paradoxical ERK activation in certain RAS mutant cell lines[22]. This review highlights the significance of RAF dimerization in MAPK signaling and the regulatory mechanisms of RAF dimers. This paper also summarizes the progress in developing RAF dimer inhibitors for RAS mutant tumors, explicitly focusing on resistance mechanisms and therapeutic strategies to combat resistance.

2. Two main strategies for targeting oncogenic RAS

2.1. *Targeting oncogenic RAS proteins directly*

Two main strategies for targeting oncogenic RAS signaling have been identified: direct and indirect (Fig. 1). Since KRAS mutations account for the most significant proportion of RAS, current direct targeting strategies focus on KRAS mutations. There are many obstacles to directly targeting oncogenic KRAS proteins because of their strong affinity for GTP and the lack of small molecule drug binding pockets[23]. KRAS was considered an "undruggable" target until KRAS G12Ci made breakthroughs in recent years. The KRAS G12C covalent inhibitors, such as sotorasib and adagrasib, can bind to the switch II pocket of KRAS G12C-GDP and effectively lock it in the inactive state[24]. Recent clinical success in targeting KRAS G12C has inspired scientists to search for novel strategies targeting alternative KRAS mutants. MRTX1133, a selective and non-covalent KRAS G12D inhibitor, potently inhibits both the activated and inactivated states of KRAS G12D, exhibiting more than 1000-fold more excellent antitumor activity against mutant KRAS G12D tumor cells than against wild-type (WT) KRAS tumor cells[25]. However, its bioavailability is currently limited and requires further optimization[26]. In addition to small molecule inhibitors, several novel therapeutics targeting specific KRAS mutations, such as the KRAS G12D degrader (ASP3082)[27], KRAS G12D siRNAs (siG12D-LODER[28] and iExosomes[29]) and KRAS G12D/V-specific T cell receptor (TCR)-engineered T cell therapy[30], have also been developed and entered clinical trials (Table 1). These mutant-specific KRAS therapies exhibit reduced toxicity toward normal cells but require individualized development of each KRAS mutant and may be more susceptible to drug resistance.

Conversely, pan-KRAS therapies aim to encompass a broader patient population, including those who display resistance to mutant-specific KRAS therapies. The development of pan-KRASi is underway. For instance, the recently reported pan-KRASi BI-2865 can bind to KRAS-GDP and block GTP-GDP nucleotide exchange, thereby inhibiting WT KRAS and various KRAS mutants such as G12 A/C/D/F/V/S and G13C/D, and sparing NRAS and HRAS[31]. Several pan-KRAS therapies have already been tested in clinical trials[32,33]. RSC-1255 inhibits oncogenic KRAS signaling by inhibiting vacuolar ATPase activity, showing heightened potency against the KRAS G13D and G12V mutants[32]. A phase I trial of RSC-1255 in advanced solid tumor malignancies is ongoing (NCT04678648). Cancer vaccines have emerged as another promising approach for targeting pan-KRAS mutant

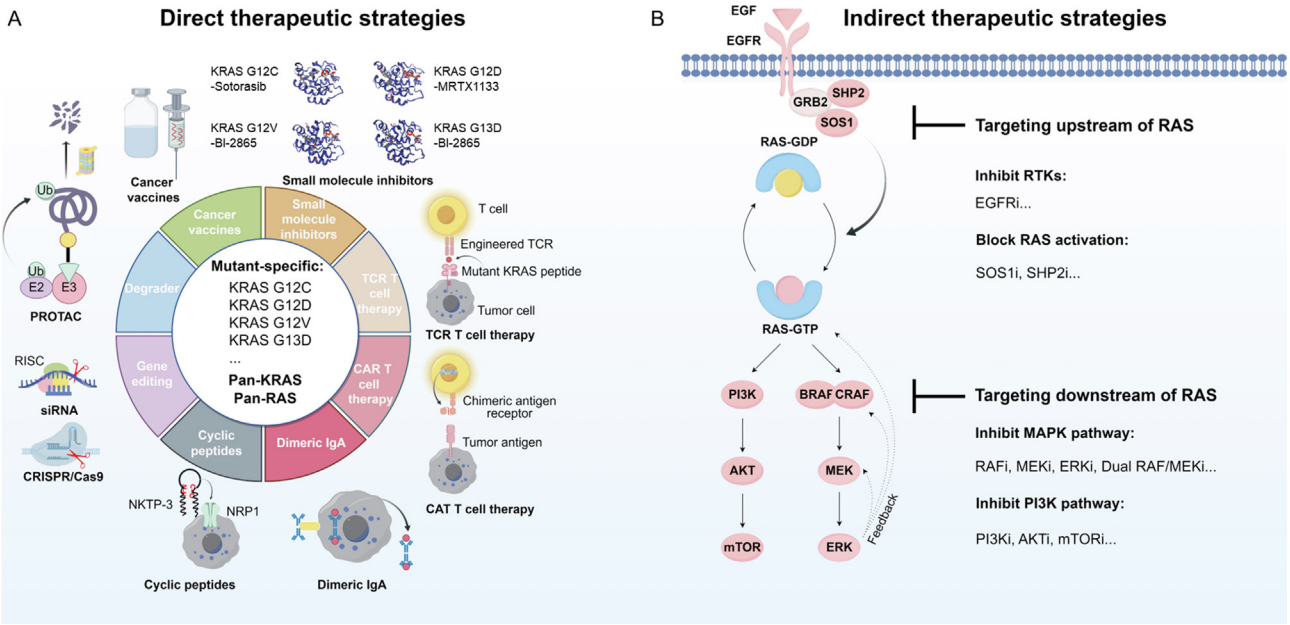

Figure 1 Strategies for targeting oncogenic RAS signaling. (A) Direct targeting strategies are categorized into targeting mutant-specific KRAS and targeting pan-KRAS or pan-RAS, including small molecule inhibitors (KRAS G12C−sotorasib complex, PDB: 6OIM; KRAS G12D−MRTX1133 complex, PDB: 7T47; KRAS G12V−BI-2865 complex, PDB: 8AZZ; KRAS G13D−BI-2865 complex, PDB: 8B00.)[25,53], PROTAC[27,54], gene editing therapy (siRNA[29], CRISPR/Cas9[55,56]), TCR T-cell therapy[57,58], CAR T-cell therapy[59,60], cancer vaccines[33], mutant KRAS-specific dimeric IgA[61], and cyclic peptides[62,63]. (B) Indirect targeting strategies include targeting upstream of RAS (such as RTKs and SHP2/SOS1) and targeting downstream of RAS (such as RAF-MEK-ERK and PI3K-AKT-mTOR). PROTAC, proteolysis targeting chimeras; TCR, T-cell receptor; CAR, chimeric antigen receptor; RISC, RNA-induced silencing complex; NRP-1, receptor neuropilin-1.

tumors. One such mRNA cancer vaccine, mRNA-5671/V941[34], has been developed to specifically target the KRAS G12C/D/V and G13D mutants, exhibiting potential immunostimulatory and antitumor effects (NCT03948763). Another cancer vaccine, ELI-002[33], comprises amphiphile-modified mutant KRAS peptides and amphiphile-modified CpG oligonucleotides. This vaccine activates the autoimmune system by delivering antigenic peptides into the lymphatic system, eliminating tumor cells. Its clinical trial program includes ELI-002 2P (2 peptides, including KRAS G12D/R, NCT04853017) and ELI-002 7P (7 peptides, including KRAS G12 A/C/D/R/S/V and G13D, NCT05726864). Pan-RAS therapies have also been proposed to inhibit all three RAS isoforms (KRAS, HRAS, and NRAS). However, the tolerability of pan-RAS therapies may be a novel clinical issue, as adult mice die from ablation of all three RAS isoforms[35]. RMC-6236 can inhibit RAS activation by inducing the formation of a tri-complex of cyclophilin A and RAS[36]. Thus, RMC-6236 can result in significant and sustained regressions in tumors with WT and mutant RAS variants, particularly those harboring mutations at the KRAS G12 locus. RMC-6236 is currently being evaluated in patients with RAS mutant solid tumors (NCT05379985), and the preliminary results indicated that the objective response rate (ORR) was 36% in KRAS G12X mutant patients with pancreatic cancer and NSCLC[37]. However, further studies are needed to fully investigate the potential of pan-KRAS and pan-RAS therapies to overcome drug resistance, and attention must be given to the increased toxicity, especially in combination therapies.

2.2. *Targeting oncogenic RAS signaling indirectly*

Targeting oncogenic RAS signaling indirectly through inhibiting key related targets in both the upstream and downstream of mutant RAS represents a significant strategy[4]. SOS1 and SHP2 are crucial targets upstream of RAS[38,39]. Inhibition of these two targets has demonstrated antitumor activity against pan-KRAS mutants. Hence, they are also referred to as indirect pan-KRASi[35]. With the potential to inhibit reactivation of the RAS/MAPK pathway, multiple combination regimens are being evaluated in clinical trials, such as BI 1701963[40], TNO155[41], and RMC-4630[42]. Additionally, extensive efforts have been made to develop inhibitors that block major kinases within the downstream pathway of RAS, such as RAF-MEK-ERK and PI3K-AKT-mTOR. However, the intricate crosstalk between signaling pathways poses challenges for indirect targeting strategies. Single-agent multi-target inhibitors may hold promise. The novel dual RAF/MEK inhibitor VS-6766 has emerged as a potential therapeutic option for various RAS-mutated tumors[43]. Combinatorial therapies based on these inhibitors, including vertical and horizontal combinations, have shown remarkable efficacy in clinical trials and could overcome resistance. Vertical combinations can induce more effective and durable inhibition of MAPK signaling by targeting multiple targets of the RAS/MAPK pathway[44-46]. Horizontal combinations that simultaneously target RAS signaling and other RAS-related pathways may result in increased antitumor activity, for example, by simultaneously inhibiting the PI3K pathway or

Table 1 Therapies directly targeting oncogenic KRAS signaling in clinical trials.

Therapy	Target	Mechanism	Phase	Trial number	Ref.
KRAS G12C					
Sotorasib (AMG510)	$KRAS^{G12C}$	Covalent inhibitor	Approved	NCT04303780, NCT03600883, NCT04185883, NCT04380753	8
Adagrasib (MRTX849)	$KRAS^{G12C}$	Covalent inhibitor	Approved	NCT04330664, NCT03785249, NCT04613596, NCT04793958	9
Divarasib (GDC-6036)	$KRAS^{G12C}$	Covalent inhibitor	III	NCT04449874, NCT04302025, NCT03178552, NCT04589845, NCT04929223	64
JDQ443	$KRAS^{G12C}$	Covalent inhibitor	III	NCT05999357, NCT05329623, NCT05358249, NCT04699188, NCT05714891, NCT05445843, NCT05132075	41
LY3537982	$KRAS^{G12C}$	Covalent inhibitor	III	NCT04956640, NCT06119581	65
D-1553	$KRAS^{G12C}$	Covalent inhibitor	II	NCT05383898, NCT05492045, NCT04585035, NCT05379946	66
JAB-21822	$KRAS^{G12C}$	Covalent inhibitor	II	NCT05009329, NCT05002270, NCT05194995, NCT05276726, NCT06008288, NCT05288205	67
GFH925	$KRAS^{G12C}$	Covalent inhibitor	II	NCT05756153, NCT05005234, NCT05497336, NCT05504278	68
BI 1823911	$KRAS^{G12C}$	Covalent inhibitor	I	NCT04973163	69
JNJ-74699157	$KRAS^{G12C}$	Covalent inhibitor	I	NCT04006301	70
MK-1084	$KRAS^{G12C}$	Covalent inhibitor	I	NCT05067283	71
BPI-421286	$KRAS^{G12C}$	Covalent inhibitor	I	NCT05315180	72
GH35	$KRAS^{G12C}$	Covalent inhibitor	I	NCT05010694	73
RMC-6291	$KRAS^{G12C}$	Form a tri-complex with cyclophilin A and RAS	I	NCT06128551, NCT05462717	74
KRAS G12D					
RMC-9805	$KRAS^{G12D}$	Form a tri-complex with cyclophilin A and RAS	I	NCT06040541	75
HRS-4642	$KRAS^{G12D}$	Small molecule inhibitor	I	NCT05533463	76
MRTX1133	$KRAS^{G12D}$	Non-covalent inhibitor	I/II	NCT05737706	25
ASP3082	$KRAS^{G12D}$	Degraders	I	NCT05382559	27
siG12D-LODER	$KRAS^{G12D}$	siRNA	II	NCT01188785, NCT01676259	28
iExosomes	$KRAS^{G12D}$	Exosomes with siRNA	I	NCT03608631	29
LUNA18	$KRAS^{G12D}$	Cyclic peptide inhibitor	I	NCT05012618	63
Anti-KRAS G12D mTCR PBL	$KRAS^{G12D}$	TCR-engineered T cell therapy	I/II	NCT03745326	30
KRAS G12V					
Anti-KRAS G12V mTCR PBL	$KRAS^{G12V}$	TCR-engineered T cell therapy	I/II	NCT03190941	30
KRAS G12V-specific TCR transduced autologous T cells	$KRAS^{G12V}$	TCR-engineered T cell therapy	I/II	NCT04146298	–
YK0901 immunotherapy	$KRAS^{G12V}$	TCR-engineered T cell therapy	I	NCT05933668	–

Table 1 (*continued*)

Therapy	Target	Mechanism	Phase	Trial number	Ref.
FH-A11KRASG12V-TCR	$KRAS^{G12V}$	TCR-engineered T cell therapy	I	NCT06043713	–
Pan-(K)RAS					
RSC-1255	$KRAS^{G12V}$, $KRAS^{G13D}$	Inhibiting vacuolar-ATPase	I	NCT04678648	32
mRNA-5671/V941	$KRAS^{G12C/D/V}$, $KRAS^{G13D}$	Cancer vaccines	I	NCT03948763	34
ELI-002 2P	$KRAS^{G12D/R}$	Cancer vaccines	I/II	NCT05726864, NCT04853017	33
RMC-6236	$KRAS^{G12D/V}$, $KRAS^{G13D}$, $KRAS^{Q61K}$, RAS^{WT}	Form a tri-complex with cyclophilin A and RAS	I/II	NCT06128551, NCT06162221, NCT05379985	36,37

PBL, peripheral blood lymphocyte; TCR, T-cell receptor.

the CDK4/6 pathway[47,48]. These combinations have demonstrated a synergistic impact in preclinical models, albeit with the need to strike a delicate equilibrium between efficacy and tolerability in clinical trials. As a critical target in the MAPK pathway, RAF kinase inhibitors have undergone several generations of iterations. Based on in-depth studies of the RAS-RAF activation process, RAF dimer inhibitors have been developed. Notably, several clinical trials have demonstrated their potential as mono- and combination therapies in treating patients with RAS mutant tumors[49-52].

3. Importance of RAF dimerization in RAS/MAPK signaling

3.1. Structural and functional features of RAF kinases

3.1.1. Structure of RAF kinases

RAF kinases are composed of an N-terminal regulatory region (NTR) and a C-terminal kinase structural domain (KD) and consist of three conserved regions (CRs). The NTR includes a RAS-binding structural domain (RBD), a cysteine-rich structural domain (CRD), and a serine/threonine-rich region (Fig. 2A)[77]. The RAF protein exists as a monomer in the cytoplasm in an inactive conformation, which is inhibited by intramolecular interactions between the NTR and KD. Upon activation of RTK, RAS-GTP recruits and attaches to the RAF-RBD, leading to phosphorylation of specific residues[78]. This enables the anchoring of the RAF protein to the cell membrane and causes lateral dimerization and activation of RAF kinases[79].

The RAF protein has a typical kinase domain structure that consists primarily of the N lobe, the C lobe, and the activation loop. The activation loop connects the two lobes and is located near the ATP binding site[78]. Typically, this activation loop encompasses 20−30 amino acid residues, starting with a DFG sequence (Asp-Phe-Gly) and ending with an APE sequence (Ala-Pro-Glu)[80]. The DFG-Asp side chain is near the ATP binding site in active RAF kinases, resulting in the DFG-in conformation. Conversely, the DFG-Asp side chain is distant from the ATP binding site in inactive RAF kinases, leading to the denoted DFG-out conformation[81]. The conformations can also be defined based on the DFG-Phe residues[82]. Moreover, the N lobe contains five β-folds and an αC-helix[78], wherein a lysine in the β3 chain (β3-Lys) can create a "salt bridge" with a glutamic acid in the αC-helix (αC-Glu). This interaction is characteristic of the αC-helix-in conformation in active RAF kinase[83]. The RAF kinase adopts the αC-helix-out conformation without this interaction, which is inactive. The αC-helix-in conformation transition is essential for the activation of RAF kinase and impacts the function of RAFi.

3.1.2. The three RAF isoforms play different roles in RAS signaling

Despite sharing common activators and substrates, the three isoforms of RAF (ARAF, BRAF, and CRAF/RAF1) exhibit distinct characteristics in kinase activity, regulatory mechanisms, and functional roles[86]. RAF kinases play an essential intermediate role in the MAPK signaling cascade and undergo several critical events for activation. These processes include recruitment to the cell membrane and activation *via* RAS activation, RAF dimerization, phosphorylation at distinct structural domains, attachment to scaffold protein complexes, and regulation of activity by chaperone proteins[87]. Research on RAF knockout mice indicated that the three RAF isoforms have distinct physiological functions during development[88]. Nonetheless, the specific mechanisms through which these isoforms contribute to the progression of RAS mutant tumors in different tumors are still poorly understood. BRAF exhibits the highest kinase activity in activating downstream ERK signaling, whereas ARAF and CRAF exhibit comparatively lower kinase activity. Notably, CRAF could play a crucial role in the progression of RAS mutant tumors[88,89]. CRAF, rather than BRAF, is responsible for triggering ERK reactivation[90]. Nevertheless, selective CRAF inhibition promotes paradoxical activation due to RAF dimerization[91], whereas suppressing CRAF has been found to enhance the efficacy of MEKi in KRAS mutant tumors[90]. Limited studies have been conducted on the function of ARAF, but recent investigations have revealed its capacity to regulate RAS activity[92]. Specifically, ARAF competitively hinders the binding of the GAP protein NF1 to RAS, thereby impeding the negative regulatory impact of the GAP protein on RAS. Importantly, this regulatory function of ARAF is independent of its kinase activity. Cells exhibiting elevated ARAF expression show an augmented baseline level of RAS-GTP, leading to drug resistance. There is a need to gain a broader understanding of the functions of RAF isoforms in different types of tumors, which may provide valuable perspectives on the distinct reactions of RAS mutant tumors to particular treatment approaches.

3.2. RAF dimerization in normal and oncogenic RAS/RAF signaling

MAPK signaling in both normal cells (wild-type RAS/RAF) and RAS mutant tumor cells depends on RAF dimers. RAF dimerization plays a vital role in MAPK signaling reactivation and drug

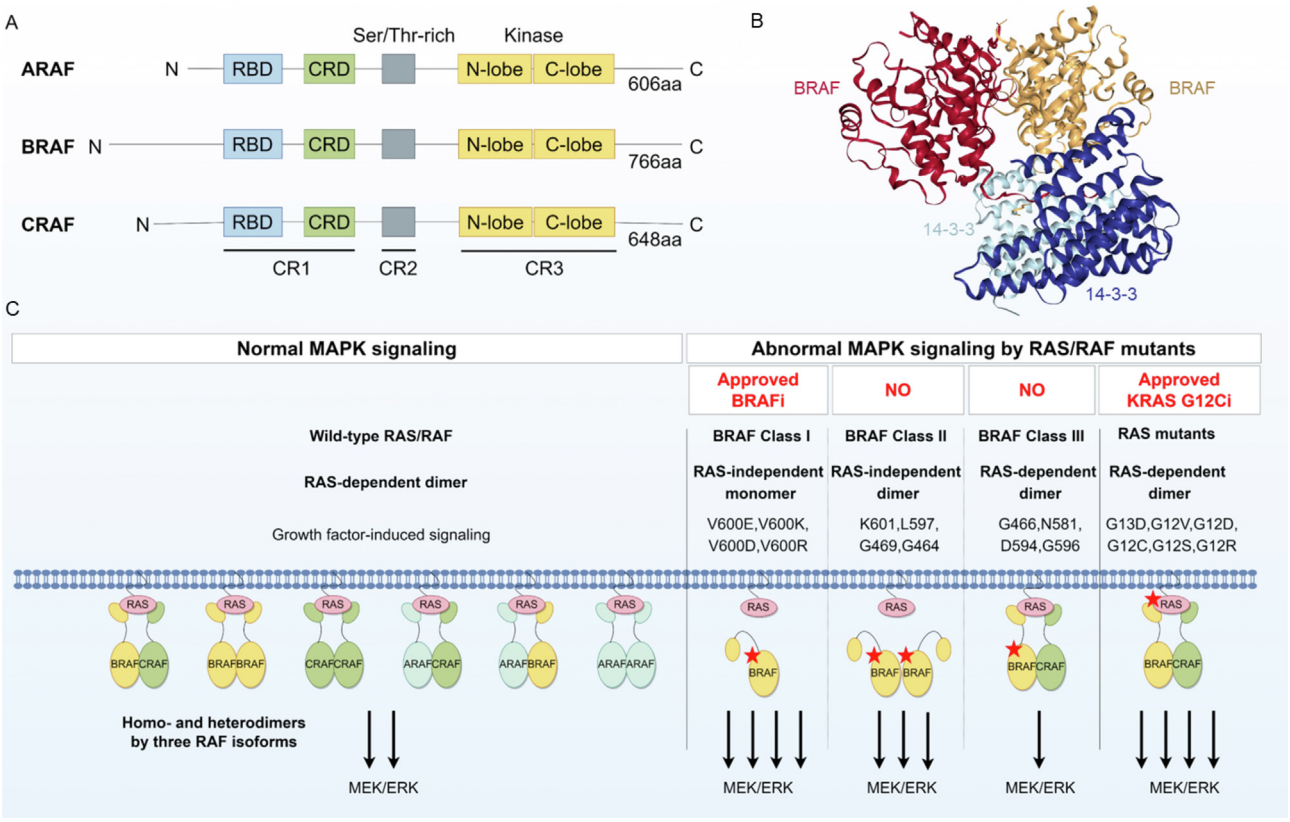

Figure 2 Structural and functional features of RAF dimers. (A) Domain structures of the three RAF isoforms. There are three conserved regions (CRs) of RAF kinases. CR1 contains a RAS-binding structural domain (RBD) and a cysteine-rich structural domain (CRD). CR2 is a serine/threonine-rich region, and CR3 contains a kinase structural domain. (B) The structure of the BRAF dimer bound to 14-3-3 (PDBID: 6U2H)[84]. Dimeric 14-3-3 promotes BRAF dimer formation (side-to-side), and the image was generated with the NGL Viewer[85]. (C) The role of RAF dimers in MAPK signaling in normal and RAS/RAF mutant cells. All three RAF isoforms can form RAF homo or heterodimers (six in total). BRAF and CRAF dimers play important roles in MAPK signaling and exhibit more excellent kinase activity than ARAF dimers, which were recently found to be involved in drug resistance. Both normal cells and RAS mutant tumor cells activate downstream ERK signaling through RAS-dependent RAF dimers, and RAS mutations result in sustained and significant activation of ERK signaling. BRAF class I mutations function as RAS-independent BRAF mutant monomers with the highest kinase activity. BRAF class II mutations are characterized by RAS-independent BRAF mutant dimers with moderate BRAF kinase activity. BRAF class III mutations tend to form heterodimers with wild-type CRAF, activated by RAS-dependent RAF dimers with the lowest kinase activity. The red pentagrams represent mutated RAS/RAF proteins.

resistance. However, only a few RAF mutants act through RAF dimers, and monomeric BRAF V600 locus mutations account for most of these mutations. Breakthroughs were made in the field of BRAF V600E inhibitors a decade ago, which have been approved for clinical use[93]. Therapeutic strategies for other RAF dimer-driven RAF mutant tumors and RAS mutant tumors are still under investigation.

3.2.1. Structure and detection of RAF dimers

In addition to RAF dimers, dimerization was also observed in RAS, MEK, and ERK kinases, highlighting the importance of dimerization in the MAPK signaling pathway[94]. However, the specific mechanisms and functions of these dimers remain unclear. The crystal structures of RAF dimers have been recently revealed, providing insights into the structural foundation of kinase activation and drug development (Fig. 2B)[13,84,95,96]. Two RAF protomers combine to form a RAF dimer in a lateral arrangement (side-to-side). The dimer interface (DIF) contains a specific cluster of residues essential for dimerization and presents potential therapeutic targets for RAF dimer inhibition[79,97]. The conformation of DIF can be altered by key residues that, for example, inhibit RAF dimerization (BRAF R509H and CRAF R401H) or promote RAF dimerization (BRAF E586K and CRAF E478K)[98]. Recent studies on RAF dimerization have focused on BRAF and CRAF, as ARAF is the least inclined to dimerize because of its unique structural characteristics and lack of stability in DIF[98,99].

Current methods for detecting and studying RAF dimers are still under development. The coimmunoprecipitation (co-IP) method is widely used as the classical experimental approach for investigating the interaction between RAF dimers by tagging RAF proteins differently. Co-IP can only probe RAF protein interactions solely in cell-free lysates, which requires the level of RAF expression and stability of the RAF dimers. Alternatively, proximity ligation assays can be employed to detect RAF dimerization in fixed cells[98,100]. Additionally, Lavoie et al. proposed a biosensor system utilizing bioluminescence resonance energy transfer to detect RAF dimers in living cells[101]. The split luciferase system has emerged as a novel method for investigating RAF dimers in living cells. This system involves the fusion of RAF proteins with various luciferases, such as firefly (Photinus)

luciferase, click beetle (Pyrearinus) luciferase, and Nanoluc luciferase[102-104]. When the target fusion protein interacts and brings two luciferase fragments close together, the split luciferase protein undergoes reorganization, activating catalytically active luciferase. The split luciferase system allows researchers to study the real-time dynamics of RAF dimers within living cells.

3.2.2. *RAF dimerization in normal cell signaling*

All three RAF isoforms can form homo and heterodimers, which are crucial for kinase activity under normal physiological conditions[79]. The prevailing belief is that when RAS-GTP is activated, and scaffold complexes such as SHOC2-PP1C are present, they recruit autoinhibited RAF monomers to the cell membrane. This process promotes intracellular colocalization and ultimately facilitates RAF dimerization[105,106]. The RAF dimer exhibits functional asymmetry, where one protomer can function as an activator to enhance the activity of the other protomer, termed *trans*-activation[107]. Despite variations in their capacity to induce ERK signaling, full activation of all RAF isoforms depends on RAF dimerization[21]. Linda et al. observed endogenous BRAF/CRAF heterodimers under physiological conditions, which are the predominant RAF dimers in normal cells[108]. Additionally, the BRAF/CRAF heterodimers were found to be negatively regulated by ERK signaling (by inducing phosphorylation of the BRAF T753 site). Remarkably, even the kinase-inactive form of BRAF can activate ERK signaling by forming a dimer with CRAF[108], underscoring the importance of RAF dimerization in RAF activation.

3.2.3. *RAF dimerization in oncogenic RAS signaling*

In RAS mutant tumors, RAF dimerization is critical for MEK-ERK signaling and contributes to drug resistance to MAPK pathway inhibitors. Although a complete understanding of the formation preferences of RAF dimers and their specific mechanisms remains elusive, it is believed that different RAF dimers may uniquely contribute to the progression of different types of tumors. In 2001, Weber et al. discovered that active RAS G12V could stimulate BRAF/CRAF heterodimer formation[109]. The BRAF/CRAF heterodimer plays a dominant role in RAS signaling due to its high kinase activity compared to other RAF dimers[98]. It is closely associated with treatment response and has significant clinical implications[110]. Quantitative proteomic studies have further revealed that ARAF/CRAF dimers are more abundant than BRAF/CRAF dimers in KRAS mutant tumors[88]. Notably, the tumorigenesis process depends on the dimerization of CRAF rather than on its activity[88]. In KRAS mutant tumors, MEKi promotes BRAF/CRAF dimer formation and induces a rebound in MEK-ERK signaling, leading to drug resistance[20,111]. Furthermore, recent studies on the mechanisms of RAF dimerization have focused on the regulation of RAF dimers by scaffolding proteins. Scaffolding proteins are essential for RAF dimer formation and activation, such as SHOC2[20,112]. Recent studies have drawn attention to the synthetic lethal interaction between SHOC2 and MEKi in RAS mutant tumors[113]. In these tumors, the absence of SHOC2 hinders MEKi-induced BRAF/CRAF dimers, resulting in more potent and sustained inhibition of ERK signaling[20]. Notably, SHOC2 may play an essential role in the resistance mechanisms of RAS/MAPK pathway inhibitors, such as KRAS G12Ci[114]. Scaffolding proteins provide temporal and spatial control over ERK signaling activation and may be potential targets in RAS mutant tumors.

The first-generation RAF inhibitors, which bind to one of the protomers and induce RAF dimerization, result in allosterically significant activation of the other protomer and subsequent ERK activation[77,115]. Consequently, these inhibitors are ineffective and may even be contraindicated for RAS mutant tumors. New-generation RAF inhibitors employ different strategies to target and inhibit RAF dimers, with RAF dimer inhibitors showing particular promise. Nonetheless, the existing RAF dimer inhibitors cannot completely inhibit the activity of all RAF dimers, especially homo and heterodimers of ARAF, and induce ERK reactivation by ARAF dimerization[18,116]. Therefore, to effectively inhibit ERK signaling and treat RAS mutant tumors, it is imperative to focus on targeting RAF dimers.

3.2.4. *Only a few RAF mutants act through RAF dimers*

Approximately 8% of human tumors carry BRAF mutations[81]. Mutations in the BRAF gene are predominant within the V600 motif and constitute 98.4% of the total mutations, whereas mutations within the non-V600 motif account for a mere 1.6% of the total mutations[78]. Three types of BRAF mutations are classified according to the characteristics of the mutant kinase activity and its dependence on RAS and RAF dimerization (Fig. 2C)[12,115]. BRAF class I mutations include various mutations at the BRAF V600 locus (such as BRAF V600 E/K/D/R). Unlike wild-type BRAF, which requires RAS-GTP and RAF dimerization for catalytic activation, BRAF class I mutations exhibit the highest kinase activity and function as RAS-independent BRAF mutant monomers. Monomeric BRAF V600E inhibitors, such as vemurafenib, are effective against BRAF class I mutations and have been approved for clinical use. BRAF class II mutations (such as BRAF K601, L597, G469, and G464), characterized by RAS-independent BRAF mutant dimers, exhibit moderate BRAF kinase activity. BRAF class II mutations are sensitive to RAF dimer inhibitors[115]. BRAF class III mutations (such as BRAF G466, N581, D594, and G596) with impaired kinase activity tend to form dimers with wild-type CRAF and bind tightly to RAS-GTP[117]. Thus, BRAF class III mutations are activated by RAS-dependent RAF dimers and exhibit the lowest kinase activity. These mutations are often accompanied by mutations in upstream oncogenes such as RAS and NF1, further complicating matters. Combination therapies targeting both upstream (RTKi or SHP2i) and downstream signaling (MEKi or ERKi) may be potential therapeutics for BRAF class III mutations[118].

In addition to point mutations, fusion proteins arising from translocations and in-frame deletions can also trigger continuous activation of the BRAF kinase[81,119]. These BRAF fusion proteins are predominantly observed in melanoma and gliomas[120]. They typically contain structural domains of protein kinase but lack the NTR, which is responsible for the self-inhibition of BRAF. Consequently, this absence promotes continuous RAF activation through a RAS-independent dimer[121], making the fusion proteins susceptible to inhibition by RAF dimer inhibitors[115]. In addition, BRAF in-frame deletions are most frequently observed in pancreatic and lung tumors[100]. These deletions cause a reduction in the length of the $\beta 3/\alpha$C-helix loop of the BRAF kinase, leading to the stabilization of the αC-helix-in conformation and promoting dimer formation[119]. While these deletions are not sensitive to the RAF monomer inhibitor vemurafenib, they exhibit sensitivity to RAF dimer inhibitors[100]. Chen et al. proposed that this discrepancy arises from promoting BRAF homodimer formation[100]. Nonetheless, some researchers have suggested that resistance to vemurafenib is independent of RAF dimerization but attributed to

the spatial blocking effect and conformational changes associated with the deletions[119,122].

In contrast, mutations in the ARAF and CRAF genes occur less frequently than BRAF mutations. The mutational hotspots in the ARAF and CRAF genes differ significantly from those observed in BRAF mutations[12]. A complete understanding of these mutations' activation mechanisms and consequences on MAPK signaling has yet to be achieved. Wenjing et al. discovered that ARAF S214 mutations could enhance MEK phosphorylation by forming a persistent activation dimer[92]. This process is independent of RAS activity and resembles BRAF class II mutations. CRAF fusions also act as RAF dimers and are thus resistant to RAF monomer inhibitors but sensitive to RAF dimer inhibitors[123]. Although there are specific variations, RAF dimerization plays a significant role in these RAF mutants.

4. Therapeutic strategies to target RAF dimers in RAS mutant tumors

Various approaches have been employed to target RAF dimers in tumors harboring RAS mutations (Table 2). Most presently developed RAFi bind directly to the ATP-binding site of the RAF protein, thereby impeding the activation of downstream MEK/ERK, so-called ATP competition inhibitors. Based on the conformation of the bound RAF kinase, the currently developed RAF inhibitors can be classified into three groups: type I, type I1/2, and type II RAFi (Fig. 3A–D)[22,124]. Another distinct class of RAF inhibitors are paradox breakers, which disrupt RAF dimers[125]. Furthermore, allosteric inhibitors directly targeting DIF are called DIF peptide inhibitors (type IV RAFi)[126]. Notably, type II RAFi and DIF peptide inhibitors have demonstrated preliminary antitumor efficacy against RAS mutant tumors (Fig. 4). It is imperative to devote particular attention to treating tumors with KRAS mutations. Compared to NRAS mutant tumors, KRAS mutant tumors exhibit reduced sensitivity to RAFi[5]. Among these RAFi, only type II RAFi have demonstrated activity against KRAS mutant tumors in both preclinical and clinical settings, although to a lesser degree than BRAF V600E mutations.

4.1. *Inability of type I and type I1/2 RAFi to inhibit RAF dimers*

Type I RAFi interact with RAF kinases *via* the DFG-in and αC-helix-in conformation. Type I RAFi (such as GDC0879 and SB590885) can adopt a stable, closed, and active conformation[22,127,128]. Nevertheless, these inhibitors are only effective against tumor cells that are activated by RAF monomer-driven ERK signaling (BRAF class I mutations). Type I RAFi stabilize both protomers in RAF dimers in the αC-helix-in conformation, thus significantly promoting RAF dimerization, which leads to paradoxical ERK activation[104]. RAS mutant tumor cells often show reduced sensitivity or even resistance to type I RAFi compared to cells with BRAF V600E mutations[128].

Type I1/2 RAFi, such as vemurafenib, dabrafenib, and encorafenib, can attach to RAF kinases in the DFG-in and αC-helix-out conformation. These inhibitors, usually called first-generation RAFi, show potent inhibitory effects on BRAF class I mutant tumor cells. When applied to mutant RAS and WT RAS/RAF cells, which function as RAF dimers, type I1/2 RAFi paradoxically activate ERK signaling[77]. The critical factors responsible for this could be the compounds binding to RAF kinase, alleviating RAF autoinhibition, inducing RAF dimerization, and enhancing RAS–RAF interactions[77]. Type I1/2 RAFi promote RAF dimerization, but the effect is weaker than type I and II RAFi[103,104]. Paradoxical activation of ERK occurs because of the partial disruption of the DIF caused by the drug-binding RAF protomer. This process prevents the inhibitors from binding to the other drug-free RAF protomer, called negative allostery, and even significantly activates the drug-free RAF protomer[81,115]. Increasing the concentration of type I1/2 RAFi can occupy both protomers of the RAF dimer, effectively inhibiting ERK signaling[129]. However, this is difficult to achieve in patients, resulting in poor efficacy of these drugs in RAF dimer-driven tumors.

The US Food and Drug Administration (FDA) has approved the use of vemurafenib and dabrafenib in treating metastatic melanoma in patients harboring BRAF V600 E/K mutations. While most melanoma patients experience tumor regression and prolonged survival[152], these medications seldom eradicate the tumor, and a notable percentage of patients still encounter both inherent and acquired resistance[153]. The clinical effectiveness of these medications is relatively low in nonmelanoma cancers that feature activating BRAF V600 mutations[154,155]. Paradoxical activation of ERK is a crucial reason for the poor outcome and limited efficacy of these agents in patients with RAS mutant tumors. They even trigger adverse reactions such as keratoacanthomas and cutaneous squamous cell carcinomas (SCCs) that lead to drug discontinuation, limiting their use in RAS mutant tumors[156]. The risk of progression can be reduced by inhibiting two nodes of the MAPK pathway to prevent ERK reactivation. The standard treatment for BRAF V600 E/K metastatic melanoma now involves combining type I1/2 RAFi with MEKi (such as vemurafenib and cobimetinib, dabrafenib and trametinib)[157,158]. Encorafenib has entered phase III clinical trials and has also shown therapeutic benefits when combined with MEKi[159].

4.2. *Type II RAFi show efficacy in RAS mutant tumors*

Type II RAFi are classified as compounds that adopt the DFG-out, αC-helix-in conformation. Usually, type II RAFi are also referred to as RAF dimer inhibitors. Tovorafenib (MLN2480) and naporafenib (LXH254) are typical type II RAFi. Their chemical structures and a detailed view of the conformations in complex with wild-type BRAF kinases are shown in Fig. 3E–F)[150]. The central thiazole ring of tovorafenib is positioned between Thr529 and Lys483. The bisubstituted pyrimidine ring of tovorafenib establishes hydrogen bonds with the BRAF kinase hinge (Cys532). The carbonyl oxygen forms a hydrogen bond with DFG-Asp594, and the neighboring amide nitrogen establishes a hydrogen bond with Glu501 in the αC-helix. The trifluoromethyl-substituted pyridine ring of tovorafenib can bind to the hydrophobic pocket formed by rotation of the DFG motif (DFG-out). Like in the case of tovorafenib, in the BRAF-naporafenib complex, the central substituted phenyl ring binds within the spatial region between Thr529 and Lys483. The trifluoromethyl pyridyl moiety of naporafenib occupies the hydrophobic pocket created by flipping the DFG motif (DFG-out). Naporafenib also forms hydrogen bonds with Cys532 in the kinase hinge, Glu501 in the αC-helix, and Asp594 in the DFG motif. These hydrogen bonds are postulated to contribute to the potent medicinal properties of these compounds[160]. These two inhibitors induce a DFG-out conformation, leading to a slight alteration in the relative orientation of the N-lobe and C-lobe, potentially leading to cooperative effects in both protomers in the dimer[150].

The interactions between type II RAFi and RAF kinase stabilize the αC-helix-in conformation in both RAF protomers, facilitating the formation of RAF dimers[161,162]. Indeed, despite

Table 2 Characteristics of inhibitors targeting RAF dimers.

Inhibitors	Conformation binding to RAF	Cell-free assay (IC_{50}: nmol/L)				Effects on RAF dimers (confirmed experimentally)	Preclinical effects	Phase	Ref.
		ARAF	BRAF	CRAF	$BRAF^{V600E}$				
Type II RAF inhibitors									
Tovorafenib (MLN2480)	DFG-out/αC-helix-in	2300	10.1	0.7	7.1	BRAF/CRAF, BRAF/BRAF, CRAF/CRAF	Effective in BRAF and NRAS mutants in melanoma	Phase III (NCT05566795)	103,104,130,131
Naporafenib (LXH254)	DFG-out/αC-helix-in	6.4	0.21	0.072	28	BRAF/CRAF, BRAF/BRAF, CRAF/CRAF	Effective in BRAF and NRAS mutants, moderately effective in KRAS mutants	Phase II (NCT04417621)	18,131-133
Belvarafenib (HM95573)	DFG-out/αC-helix-in	152	116	23	9	BRAF/CRAF, BRAF/BRAF, CRAF/CRAF, ARAF/BRAF, ARAF/CRAF	Effective in BRAF and NRAS mutants in melanoma	Phase II (NCT04589845)	116,132,133
Lifirafenib (BGB283)	DFG-out/αC-helix-in	5.6	32	6.7	23	BRAF/CRAF	Effective in BRAF mutants in CRC	Phase I (NCT03905148)	104,134,135
LY3009120	DFG-out/αC-helix-in	NA	9.1	15	5.8	BRAF/CRAF, BRAF/BRAF, CRAF/CRAF	Effective in BRAF, NRAS, and KRAS mutants in melanoma, CRC, lung, and pancreatic cancer	Phase I (NCT02014116)	102,103
CCT3833	DFG-out/αC-helix-in	NA	420	33	34	NA	Effective in KRAS mutants in CRC, lung, and pancreatic cancer	Phase I (NCT02437227)	81,136
BGB3245	NA	NA	NA	NA	NA	NA	Effective in BRAF and NRAS mutants in melanoma	Phase I (NCT05580770)	49,137, 138
TAK632	DFG-out/αC-helix-in	NA	8.3	1.4	2.4	BRAF/CRAF, BRAF/BRAF, CRAF/CRAF	Effective in BRAF, NRAS, and KRAS mutants in melanoma, CRC, lung, and pancreatic cancer	Preclinical	102,103,129
AZ628	DFG-out/αC-helix-in	NA	105	29	34	BRAF/CRAF, CRAF/CRAF	Effective in BRAF and NRAS mutants in melanomas, CRC, and thyroid cancer	Preclinical	81,101, 102,139
RAF709	DFG-out/αC-helix-in	NA	1.5	0.4	1	BRAF/CRAF	Effective in BRAF, NRAS, and KRAS mutants in melanomas, CRC, and lung cancer	Preclinical	132,140
BGB659	DFG-out/αC-helix-in	NA	NA	NA	NA	BRAF/CRAF, BRAF/BRAF	Effective in BRAF mutants in melanoma	Preclinical	115,141
DCC3084	DFG-out/αC-helix-in	903	71	34	2	BRAF/CRAF	Effective in BRAF and KRAS mutants in melanoma, CRC, lung, and pancreatic cancer	Preclinical	131
IHMT-RAF-128	DFG-out/αC-helix-in	NA	5.4	3.6	5.9	NA	Effective in BRAF and RAS mutants in solid tumors	Preclinical	142
REDX05358	NA	NA	NA	NA	NA	NA	Effective in BRAF, NRAS, and KRAS mutants in melanoma, lung cancer, and CRC	Preclinical	143
BDTX4933	NA	NA	NA	NA	NA	BRAF/CRAF, BRAF/BRAF,	Effective in BRAF and	Preclinical	133

(*continued on next page*)

Table 2 (*continued*)

Inhibitors	Conformation binding to RAF	Cell-free assay (IC_{50}: nmol/L)			Effects on RAF dimers (confirmed experimentally)	Preclinical effects	Phase	Ref.
		ARAF	BRAF	CRAF	$BRAF^{V600E}$			
					CRAF/CRAF	NRAS mutants in melanoma		
Paradox breakers								
PLX8394	DFG-in/αC-helix-out/R506-out	NA	14	23	3.8	BRAF/CRAF, BRAF/BRAF	Effective in BRAF mutants in Phase I (NCT02428712) melanoma and CRC	125,141,144
PLX7904	DFG-in/αC-helix-out/R506-out	NA	140	91	4.2	BRAF/CRAF	Effective in BRAF mutants in Preclinical melanoma and CRC	22,103, 144,145
Compound Ia	NA	NA	<5E-10	NA	1.77E-09	BRAF/BRAF	Effective in BRAF mutants in Preclinical melanoma	146,147
Hybrid 6	DFG-out/αC-helix-in/R506-out	NA	56	NA	48	BRAF/BRAF	Effective in BRAF mutants in Preclinical melanoma	148
DIF peptide inhibitors								
DI1	NA	NA	NA	NA	NA	BRAF/CRAF, BRAF/BRAF	Effective in KRAS mutants in Preclinical lung cancer	98
Pep17	NA	NA	NA	NA	NA	BRAF/BRAF	Effective in NRAS mutants in Preclinical melanoma	126
Braftide	NA	NA	364	NA	NA	BRAF/CRAF, BRAF/BRAF	Effective in KRAS mutants in Preclinical CRC	149

DIF, dimer interface; IC_{50}, half maximal inhibitory concentration; CRC, colorectal cancer; NA, not available.

differences in the degree and preference for inducing RAF dimerization, almost all ATP competitive inhibitors facilitate the formation of RAF dimers in a RAS-dependent manner[101]. However, due to the low negative allostery, type II RAFi potently bind to both RAF protomers and repress the kinase function of these RAF dimers[102,116]. RAF dimerization was observed after 10 min of exposure to most inhibitors, with peak formation occurring after 4−6 h[103]. Nevertheless, the suppressive impact of type II RAFi on kinase activity does not always correlate with the promoting effect of RAF dimers[103].

To overcome the paradoxical ERK activation in RAS mutant tumors, inhibiting the kinase activity of all RAF isoforms, including RAF monomers and dimers, could be an essential strategy. Type II RAFi are generally considered pan-RAF inhibitors because they theoretically bind to RAF monomers and dimers with similar potency and inhibit their kinase activity[102,134]. Therefore, these compounds are not expected to induce paradoxical activation of ERK signaling. These inhibitors have demonstrated a mild prophosphorylation effect on MEK and ERK only at very low concentrations[22], while higher concentrations of the compound occupying both RAF protomers can inhibit their activation potently[102,116]. Recent studies have shown that some type II RAFi potently inhibit BRAF and CRAF monomers and dimers, while their activity against ARAF is relatively weak[18,116,150], which could be the critical mechanism for ERK signaling reactivation and drug resistance. In addition, type II RAFi can have toxic effects on normal cells due to their extensive inhibition, although they inhibit mutant RAF monomers and dimers more potently than the wild-types[22]. Consequently, additional research and clinical investigations are necessary to assess their therapeutic index fully.

Some type II RAFi, such as TAK632[129], AZ628[139], RAF709[140], and DCC3084[131], are currently under investigation in preclinical research. These inhibitors have shown promising antitumor effects on tumor cells with KRAS and NRAS mutations. Other type II RAFi, such as tovorafenib[50], naporafenib[163], belvarafenib (HM95573)[116], lifirafenib (BGB283)[164] and LY3009120[165], have undergone clinical trials. These agents have demonstrated initial clinical benefits as monotherapy or when combined with MEKi or ERKi in individuals with KRAS and NRAS-mutated tumors. Furthermore, several multikinase drugs, such as sorafenib and regorafenib, can be classified as type II RAFi[132]. Screening for inhibitors that inhibit RAF dimers from multitarget compounds is also a new direction[101], although the extent of their effectiveness and toxicity in RAS mutant tumors still requires further substantiation. In addition, ponatinib, a tyrosine kinase inhibitor targeting BCR-ABL, adopts a unique conformation known as αC-helix-CENTRE[166]. Building upon the insights gained from ponatinib, Xiomaris et al. developed a novel BRAF dimer inhibitor called ponatinib hybrid inhibitor 1 (PHI1)[166]. This inhibitor demonstrates second-site positive cooperativity within the BRAF dimer, resulting in increased suppression of the second protomer when the first protomer is already bound. However, PHI1 shows limited efficacy in RAS mutant tumors. Furthermore, although the effects of some pan-RAF inhibitors on RAF dimerization have not been determined, such as exarafenib[167] and XL281[168], these agents have progressed to clinical trials and exhibited preliminary antitumor activity in NRAS and KRAS mutant tumors.

4.3. *Paradox breakers offer new opportunities*

RAF dimer disruption is a novel strategy for overcoming paradoxical ERK signaling. These RAFi are called “paradox breakers”, exemplified by PLX7904 and PLX8394, which have

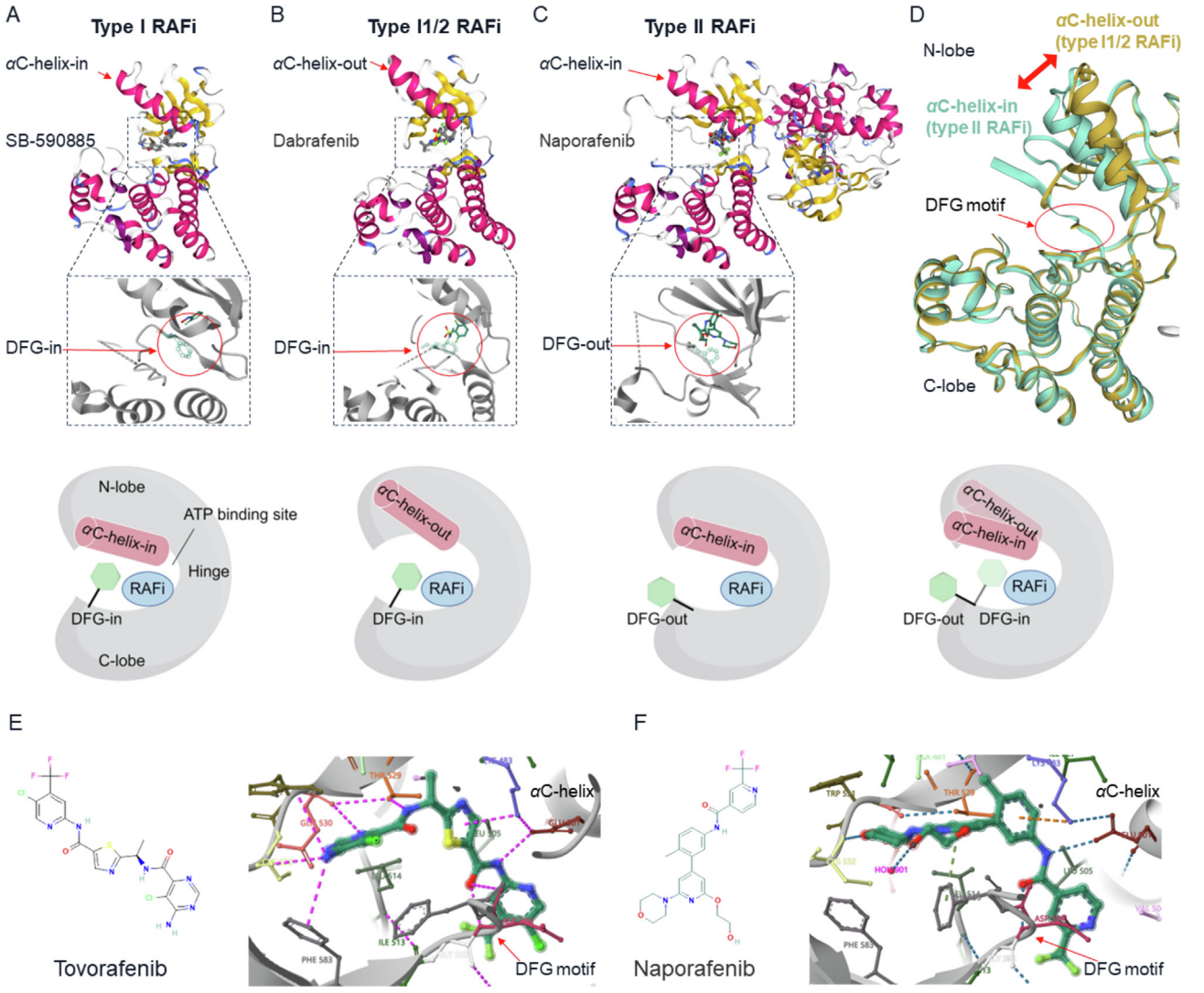

Figure 3 The conformations of RAF kinases bound to type I, I1/2, and II RAFi. The three main conformations of the RAF kinase domain are shown in (A−D). The crystal structure of each conformation is shown above. Below the crystal structure is a schematic representation showing the conformations of the DFG motif (green) and the αC-helix (red). (A) SB-590885 (type I RAFi) interacts with BRAF kinase in the DFG-in/αC-helix-in conformation (PDB: 2FB8). (B) Dabrafenib (type I1/2 RAFi) binds to BRAF kinase in the DFG-in/αC-helix-out conformation (PDB:5CSW). (C) Naporafenib (type II RAFi) binds to both BRAF protomers in the DFG-out/αC-helix-in conformation (PDB: 8F7P)[150]. (D) Comparison of the conformations of type I1/2 RAFi (teal for dabrafenib, PDB: 5CSW) and type II RAFi (green for naporafenib, PDB: 8F7P) bound to BRAF kinase. The DFG-Phe residues point toward the αC-helix in the DFG-in conformation, and the DFG-Phe residues point away from the αC-helix in the DFG-out conformation. The image was created with SWISS-MODEL[151]. (E−F) Structures of two typical type II RAFi currently under clinical investigation (tovorafenib and naporafenib), as well as their specific conformations for binding to the WT BRAF kinase (PDB: 8F7O and 8F7P)[150].

undergone structural modifications derived from vemurafenib[144]. Paradox breakers occupy one active site of RAF dimers, causing a conformational change with DFG-in, αC-helix-out, and R506-out[22]. These agents disrupt the DIF of RAF kinases, particularly BRAF/BRAF and BRAF/CRAF dimers[22,144]. This disruption prevents RAF dimerization and subsequently inhibits paradoxical ERK activation[125,144]. Notably, PLX8394 demonstrates a higher affinity for mutant dimeric BRAF (including BRAF fusions and splice variants) than for wild-type BRAF in normal cells[141]. Thus, its therapeutic index could be higher than that of type II RAFi. However, these compounds show weak effects on CRAF homodimers and ARAF dimers and even induce their activation, potentially resulting in drug resistance[141]. Since they are αC-helix-out inhibitors with negative allostery, their potency can be limited in RAS mutant tumors driven by homo and heterodimers of three RAF isoforms[22]. As predicted, both preclinical and clinical evidence suggests that paradox breakers exhibit greater efficacy in treating BRAF class I and II mutant tumors, while their effectiveness in treating BRAF class III and RAS mutant tumors is comparatively limited[141,169-171]. The same applies to recently reported paradox breakers: compound Ia, which offers good brain penetration[146,147], and hybrid 6, which adopts a DFG-out and αC-helix-in conformation[148], but neither shows potent activity against RAS mutant tumors.

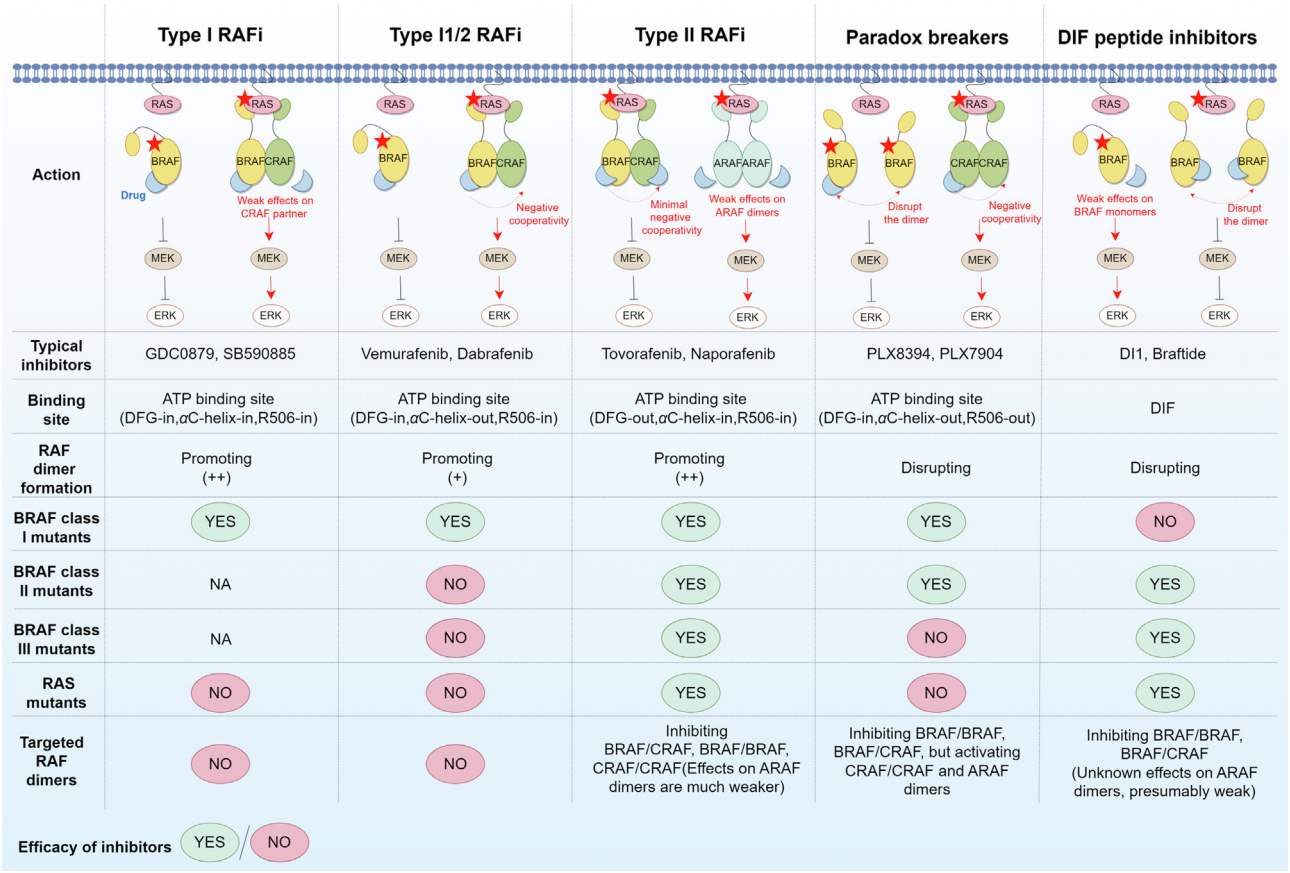

Figure 4 Role of RAF inhibitors on RAF dimers. Only type II RAFi and DIF peptide inhibitors are effective against RAS mutant tumors. These compounds effectively inhibit the kinase activity of BRAF and CRAF dimers but show weak effects on ARAF dimers, which may lead to drug resistance. The red pentagrams represent mutated RAS/RAF proteins. DIF, dimer interface.

4.4. DIF peptide inhibitors offer a promising approach

DIF peptide inhibitors, which directly target the DIF of RAF proteins, are considered promising therapeutic approaches for inhibiting RAF dimers. Most residues engaged in DIF interactions are in a contiguous sequence, enabling the design of short peptides specifically targeting DIF. Given that BRAF has the highest level of dimerization among the RAF isoforms, current efforts in developing peptide inhibitors have primarily concentrated on BRAF. In this context, Freeman et al. created a peptide inhibitor named DI1 that specifically targeted the DIF of BRAF and consists of 19 amino acids (residues 503−521)[98]. DI1 successfully dissociated the BRAF/CRAF dimer. It demonstrated remarkable efficacy in inhibiting the proliferation of NSCLC cells with KRAS and BRAF G466V mutations, both driven by RAS-dependent RAF dimers. DI1 could also inhibit pMEK signaling activated by the BRAF G464V and L597V mutations, driven by RAS-independent BRAF dimers. However, it was ineffective against tumor cells with BRAF V600E mutations, which function as BRAF monomers. In contrast, Amber et al. developed a 10-mer peptide inhibitor called braftide[149]. Braftide also targeted the DIF of BRAF and could dissociate BRAF/BRAF and BRAF/CRAF dimers. Notably, braftide selectively degraded both the BRAF and MEK kinases. Researchers posited that this dual-action mechanism may potentially delay ERK reactivation. Braftide did show activity in CRC cell lines harboring KRAS mutations, and its combination with dabrafenib effectively suppressed paradoxical ERK activation. Thus, simultaneous targeting of DIF may be synergistic with ATP competition inhibitors. Another avenue being explored involves the utilization of cyclic peptides. Compared with linear peptides, cyclic peptides exhibited enhanced binding affinity to the BRAF kinase domain, suggesting they may possess greater potency in inhibiting RAF dimers[126].

Collectively, these studies support the prospective utilization and development of novel DIF peptide inhibitors, promising for preventing the negative cooperativity and paradoxical ERK activation. The activation of kinases in all three RAF isoforms necessitates RAF dimerization, and DIF is extensively conserved across these isoforms, with only marginal disparities. Thus, inhibitors that target DIF can potentially function as pan-RAF inhibitors[107,149]. However, the impact of these developed DIF peptide inhibitors on ARAF and CRAF dimers has yet to be comprehensively elucidated. Further investigations and validations are needed to thoroughly ascertain these agents' efficacy in treating RAS mutant tumors.

5. Clinical outcomes of type II RAFi monotherapy in RAS mutant tumors

Among the research strategies for targeting RAF dimers, only type II RAFi are being evaluated in clinical trials for RAS mutant tumors (Table 3). When used as monotherapies, these inhibitors have shown initial antitumor activity in individuals with RAS-mutated tumors

Table 3 Clinical trials of inhibitors targeting RAF dimers in RAS/RAF mutant tumors.

Intervention	Indication	Cancer	Phase	Trial number	Start year	Status	Published results	Ref.
Tovorafenib (MLN2480)								
1. Tovorafenib;	BRAF/NRAS mutants	Solid tumors	I	NCT01425008	2011	Completed	YES	50
1. Tovorafenib;	MAPK alterations	LGG	I	NCT03429803	2018	Active, not recruiting	YES (Abstract)	172,173
1. Tovorafenib;	BRAF mutants	LGG	II	NCT04775485	2021	Recruiting	YES (Abstract)	175
1. Tovorafenib;	RAF mutants	LGG	III	NCT05566795	2023	Recruiting	NA	
1. Tovorafenib;	MAPK alterations	Solid tumors	I/II	NCT04985604	2021	Recruiting	NA	
2. Tovorafenib+Pimasertib (MEKi);								
1. Tovorafenib+Paclitaxel (chemotherapeutic agent);	BRAF/KRAS mutants	NSCLC	I	NCT02327169	2015	Completed	YES	clinicaltrials.gov
1. Tovorafenib+Nivolumab (anti-PD-1);	BRAF/NRAS mutants	Melanoma	I	NCT02723006	2016	Terminated	YES	clinicaltrials.gov
Naporafenib (LXH254)								
1. Naporafenib;	MAPK alterations	Solid tumors	I	NCT02607813	2016	Terminated	YES	163,176
2. Naporafenib+PDR001 (anti-PD-1);								
1. Naporafenib+LTT462 (ERKi);	BRAF/NRAS/KRAS mutants	NSCLC	Ib	NCT02974725	2017	Active, not recruiting	YES	177
2. Naporafenib+Trametinib (MEKi);		Melanoma						
3. Naporafenib+Ribociclib (CDK4/6i);								
1. Naporafenib+LTT462 (ERKi);	BRAF/NRAS mutants	Melanoma	II	NCT04417621	2020	Active, not recruiting	YES (Abstract)	52
2. Naporafenib+Trametinib (MEKi);								
3. Naporafenib+Ribociclib (CDK4/6i);								
1. Naporafenib+Dabrafenib ($BRAF^{V600E}$i)+ LTT462 (ERKi);	BRAF mutants	CRC	Ib	NCT04294160	2020	Recruiting	NA	
Belvarafenib (HM95573)								
1. Belvarafenib;	Not mentioned	Solid tumors	I	NCT02405065	2015	Completed	YES	116
1. Belvarafenib;	BRAF/NRAS/KRAS mutants	Solid tumors	I	NCT03118817	2017	Completed	YES	116
1. Belvarafenib;	BRAF mutants	Solid tumors	II	NCT04589845	2021	Recruiting	NA	
1. Belvarafenib+Cobimetinib (MEKi);	RAS/RAF mutants	Solid tumors	Ib	NCT03284502	2017	Recruiting	YES (Abstract)	178,179
2. Belvarafenib+Cetuximab (EGFRi);								
1. Belvarafenib;	NRAS mutants	Melanoma	I	NCT04835805	2021	Recruiting	NA	
2. Belvarafenib+Cobimetinib (MEKi);								
3. Belvarafenib+Cobimetinib (MEKi)+Nivolumab (anti-PD-1);								
LY3009120								
1. LY3009120;	BRAF/NRAS/KRAS mutants	Solid tumors	I	NCT02014116	2013	Terminated	YES	165
Lifirafenib (BGB283)								
1. Lifirafenib;	BRAF/NRAS/KRAS mutants	Solid tumors	I	NCT02610361	2013	Completed	YES	164
1. Lifirafenib;	BRAF/NRAS/KRAS mutants	Solid tumors	I	NCT03641586	2015	Completed	NA	
1. Lifirafenib+Mirdametinib (MEKi);	KRAS mutants	Solid tumors	Ib	NCT03905148	2019	Recruiting	YES (Abstract)	51

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Table 3 (*continued*)

Intervention	Indication	Cancer	Phase	Trial number	Start year	Status	Published results	Ref.
CCT3833								
1. CCT3833; BGB3245	BRAF/RAS mutants	Solid tumors	I	NCT02437227	2015	Completed	YES	136
1. BGB3245;	BRAF mutants	Solid tumors	I	NCT04249843	2020	Recruiting	YES	49
1. BGB3245+Mirdametinib (MEKi); PLX8394	BRAF/NRAS/KRAS mutants	Solid tumors	I/IIa	NCT05580770	2023	Recruiting	NA	
1. PLX8394;	BRAF mutants	Solid tumors	I/IIa	NCT02428712	2015	Active, not recruiting	YES (Abstract)	169
1. PLX8394;	BRAF mutants	Solid tumors	I/IIa	NCT02012231	2014	Terminated	NA	

MEKi, MEK inhibitor (same with other inhibitors); LGG, low-grade glioma; NSCLC, non-small cell lung cancer; CRC, colorectal cancer; NA, not available.

(Table 4). However, further research is still needed to exploit these agents' potential for clinical use thoroughly.

5.1. *Tovorafenib*

Tovorafenib potently blocks the kinase function of BRAF and CRAF monomers, as well as their homo and heterodimers[104,150]. Crystal structure analysis revealed that this drug binds to both protomers of the BRAF dimer, although it has a comparatively weaker impact on the ARAF monomers and dimers[150]. The efficacy of tovorafenib in treating BRAF and NRAS mutant melanoma has been shown in preclinical and clinical research[50]. Furthermore, tovorafenib possesses an excellent capability to enter the central nervous system (CNS)[130]. It has shown promising antitumor efficacy in clinical trials involving pediatric low-grade glioma (LGG) patients with RAF mutations[172-174]. The FDA granted it the "breakthrough therapy designation" in 2021, highlighting its potential in treating intracranial tumors. The drug exhibited modest efficacy in individuals with NRAS-mutated melanoma, as evidenced by a disease control rate (DCR) of 36% (5/14) and a partial response (PR) rate of 7% (1/14) (NCT01425008)[50]. The overall safety profile of tovorafenib was deemed acceptable in a cohort of 149 treated patients. A total of 68% (67/99) of the patients experienced grade $\geq$3 treatment-emergent adverse events (TEAEs), with anemia accounting for 14% (14/99) and maculopapular rash accounting for 8% (8/99). In addition, the incidence of cutaneous SCC was less than 1% (1/149), significantly lower than that of type I1/2 RAFi. Notably, tovorafenib is the only type II RAFi that has advanced to a phase III clinical trial, determining its efficacy in RAF mutant LGG (NCT05566795). However, the effectiveness of tovorafenib in RAS mutant tumors remains to be investigated.

5.2. *Naporafenib*

Naporafenib is a potent type II RAFi that effectively inhibits both monomers and dimers of BRAF and CRAF while exhibiting relatively lower activity against ARAF (approximately 30- to 50-fold less activity)[18]. Minimal paradoxical ERK activation occurs due to the occupancy of naporafenib in both protomers of the RAF dimer[160]. The efficacy of naporafenib in models with BRAF V600E and NRAS mutations has been shown *in vitro* and *in vivo*, although its activity in KRAS mutants is only moderate[18,160,180]. Furthermore, wild-type cell lines lacking RAS/RAF mutations exhibit predominant insensitivity to naporafenib, which may be attributed to their lack of dependence on the MAPK pathway. Consistent with the findings of preclinical investigations, a phase I dose-scaling trial revealed that most individuals with KRAS mutant tumors experienced disease progression following naporafenib monotherapy (NCT02607813)[176]. Only a minority of patients had stable disease (SD, 30/87), and two achieved a confirmed PR (2/87)[176]. The adverse events of naporafenib were manageable, with grade $\geq$3 TEAEs occurring in 24% of patients (21/87). While naporafenib monotherapy has limited efficacy in RAS mutant tumors, combination regimens involving napafenib have achieved significant efficacy in clinical trials.

5.3. *Belvarafenib*

Belvarafenib has been shown to selectively inhibit BRAF V600E and wild-type CRAF more potently than wild-type ARAF and

Table 4 Clinical outcomes of type II RAFi monotherapies in RAS mutant tumors.

Phase	Trial number	Pts	Administration	Efficacy in RAS mutant tumors	Safety	Ref.
I	NCT01425008	149	Single-agent tovorafenib: RP2D: 200 mg Q2D or 600 mg QW. MTD: 200 mg Q2D or 600 mg QW, over 28-day cycles.	1. NRAS-mutant melanoma in Q2D expansion phase (n = 14): DCR: 36% (5/14); PR: 7% (1/14); SD: 29% (4/14); PD: 64% (9/14).	1. Well tolerance; DLTs were observed; 2. TEAEs grade ≥3: 68% (67/99). Anemia: 14% (14/99); Maculopapular rash: 8% (8/99); SCC:< 1% (1/149).	50
I	NCT02607813	87	Single-agent naporafenib: 100−1200 mg QD or 200−800 mg BID.	1. KRAS-mutant and BRAF-mutant cancers (n = 87): DCR: 37% (32/87); PR: 2% (2/87); SD: 34% (30/87); PD: 41% (36/87).	1. Well tolerance; DLTs were observed; 2. TEAEs grade ≥3: 24% (21/87). Fatigue: 2% (2/87); Maculopapular rash: 2% (2/87); Myalgia: 2% (2/87).	163,176
I	NCT02405065 NCT03118817	135	Single-agent belvarafenib: 450 mg BID.	1. NRAS-mutant melanoma (n = 10): DCR: 60% (6/10); PR: 20% (2/10); SD: 40% (4/10). 2. KRAS-mutant CRC (n = 10): PR: 0% (0/10). 3. 1 pt with KRAS G12V-mutant sarcoma and 1 pt with KRAS G12D-mutant bladder cancer showed PR.	1. Well tolerance; 2. TEAEs grade ≥3: 33% (44/135). Rash: 3% (4/135); Dermatitis acneiform: 3% (4/135); SCC: 0% (0/135).	116
I	NCT02014116	51	Single-agent LY3009120: RP2D: 300 mg BID.	1. NRAS-mutant tumors (n = 5): DCR: 10% (1/10); PR: 0% (0/10); SD: 10% (1/10). 2. KRAS-mutant tumors (n = 17): DCR: 12% (2/17); PR: 0% (0/17); SD: 12% (2/17).	1. Bad tolerance; DLTs were observed; 2. TEAEs grade ≥3: 59% (30/51). Myalgia; Stomatitis; Fatigue.	165
I	NCT02610361	131	Single-agent lifirafenib: RP2D: 30 mg daily. MTD: 40 mg daily.	1. KRAS or NRAS-mutant tumors (n = 66): DCR: 53% (35/66); PR: 3% (2/66); SD: 50% (33/66); PD: 24% (16/66). 1 pt with KRAS-mutant endometrial cancer and 1 pt with KRAS-mutant NSCLC showed PR. But KRAS or NRAS-mutant CRC (n = 20) showed no responses.	1. Well tolerance; DLT were observed; 2. TRAEs grade ≥3:71% (68/96). Hypertension: 18% (23/131); Fatigue: 10% (13/131); SCC: 0% (0/131).	164
I	NCT04249843 (Abstract)	42	Single-agent BGB3245: MTD: 40 mg QD.	1. Objective responders included 2 pts with NRAS-mutant melanoma and 1 pt with KRAS G12D appendiceal cancer.	1. Well tolerance; DLT were observed; 2. TRAEs grade ≥3: 29% (12/42). Decreased platelet count: 7% (3/42); Rash maculopapular: 7% (3/42).	49

RP2D, recommended phase II dose; MTD, maximum tolerated dose; Q2D, once every other day; QW, once weekly; QD, once daily; BID, twice a day; DCR, disease control rate; PR, partial response; SD, stable disease; PD, progressive disease; CRC, colorectal cancer; NSCLC, non-small cell lung cancer; DLT, dose-limiting toxicity; TEAEs, treatment-emergent adverse events; SCC, squamous cell carcinoma; pts, patients.

BRAF. CRISPR-Cas9 analysis demonstrated that belvarafenib was more effective against cells expressing only BRAF or CRAF than against cells expressing only ARAF[116]. Immunoprecipitation experiments have further confirmed that this drug facilitates the dimerization of all RAF isoforms, including ARAF, and impedes kinase activity by binding to both RAF dimer protomers[116]. Moreover, it displays potent inhibitory effects on the growth of melanoma cells harboring BRAF V600E and NRAS mutations, although it induces minimal paradoxical ERK activation (compared to vemurafenib)[116]. In clinical trials involving belvarafenib monotherapy[116], a PR of 20% (2/10) was observed in individuals with NRAS-mutated melanoma, while no PR (0/10) was shown in individuals with KRAS-mutated CRC. The complicating drug resistance mechanisms in KRAS mutant CRC may account for the variable response rates[181]. However, PR was observed in two patients with sarcoma harboring the KRAS G12V mutation and bladder cancer carrying the KRAS G12D mutation, indicating its potential for treating specific KRAS mutant malignancies. The drug demonstrated good tolerability in the study involving 135 participants, and no SCC cases were observed. Furthermore, belvarafenib displayed effective blood−brain barrier penetration and exhibited potent antitumor activity against melanoma brain metastasis with BRAF/NRAS mutations in mice[182], possibly superior to that of tovorafenib[131]. Although additional clinical validation is warranted, this compound presents a potential novel therapeutic option for intracranial tumors.

5.4. *Lifirafenib*

Lifirafenib is a potent inhibitor of both pan-RAF and EGFR and can effectively inhibit the kinase activity of the three RAF isoforms with a slow off-rate[134]. The drug exhibits a marked capacity to stimulate BRAF/CRAF dimerization, although further investigation is needed to determine its impact on other types of RAF dimers[104,135]. Preclinical investigations have shown that this drug is effective in CRC cells harboring BRAF V600E or EGFR mutations but has a decreased response in cells with RAS mutations[134]. In a clinical trial involving 66 patients with KRAS- or NRAS-mutated tumors[164], lifirafenib monotherapy demonstrated a PR in a patient diagnosed with KRAS-mutated endometrial cancer and another patient diagnosed with KRAS-mutated NSCLC. Conversely, no response was observed in KRAS or NRAS mutant CRC patients ($n = 20$). The drug exhibited manageable side effects, good tolerability, and no occurrence of SCC. Additionally, the activation of EGFR feedback is a significant mechanism for the development of resistance to RAFi [183,184]. Lifirafenib has exhibited the capacity to hinder EGFR feedback activation and achieve sustained suppression of pERK[134]. Although the efficacy of EGFR inhibition may contribute to its effectiveness, the optimal therapeutic window achievable through combined RAF/EGFR inhibition has yet to be determined.

5.5. *LY3009120*

As a pan-RAF inhibitor, LY3009120 exhibits comparable affinities for ARAF, BRAF, and CRAF[100,102]. This compound stimulates homo and heterodimerization of BRAF and CRAF and subsequently binds to both protomers of the RAF dimer and prevents its kinase activity[102]. LY3009120 demonstrates reduced paradoxical ERK activation in RAS mutant tumor cells compared to RAF monomer inhibitors[102]. LY3009120-induced paradoxical ERK activation does not rely on classical RAS proteins (H/N/KRAS) but does require the MRAS/SHOC2 complex[185]. These findings indicate that upstream scaffolding protein complexes play a significant role in MAPK pathway signaling. In preclinical research, LY3009120 demonstrated remarkable effectiveness against tumors harboring BRAF, KRAS, or NRAS mutations[102,180]. Unexpectedly, the phase I clinical trial for LY3009120 was prematurely halted because of its inadequate clinical effectiveness (NCT02014116)[165]. The trial enrolled a total of 51 individuals diagnosed with BRAF- or RAS-mutated tumors. Surprisingly, no one in the study exhibited a complete or partial response, and only eight individuals (15.7%) displayed stable disease. The researchers suspected poor patient selection might have contributed to these disappointing outcomes. In addition, the short half-life and limited tumor-targeting ability of LY3009120 resulted in ineffective inhibition of pERK within the tumor microenvironment. The clinical side effects of LY3009120 must also be considered. Off-target effects may restrict the dosage of LY3009120 since it selectively affects a wide range of protein kinases, such as p38, ephrin receptors, and JNK[102].

6. Mechanisms of resistance and combination therapies

The understanding of resistance mechanisms to type II RAFi is relatively limited compared to that of KRAS G12Ci. Insights from the resistance mechanisms of KRAS G12Ci can help explain from multiple perspectives how RAS mutant tumor cells escape RAS/MAPK signaling pathway inhibition. Reactivation of the MAPK pathway and activation of other alternative pathways are common mechanisms leading to resistance to MAPK pathway inhibitors. These findings have implications for other inhibitors targeting the MAPK pathway, prompting researchers to explore comparable adaptive strategies.

6.1. *Insights from the KRAS G12Ci*

6.1.1. *Mechanisms of resistance to KRAS G12Ci*

While there are variations in the specific resistance mechanisms across different tumor types, reactivation of the RAS/MAPK pathway is a prevalent cause of resistance to KRAS G12Ci[186]. Jenny et al. reported that shortly after KRAS G12C inhibition, certain quiescent cells can generate new KRAS G12C. These mutant proteins can maintain the active, drug-insensitive state by activating EGFR and aurora kinase A signaling[187]. This rapid adaptive mechanism leads to predominantly partial responses to KRAS G12Ci in patients with NSCLC. A study of 38 patients (primarily NSCLC and CRC) receiving adagrasib showed that 45% (17/38) of patients demonstrated resistance[188]. The main resistance mechanisms can be classified into four groups: i) KRAS alterations, such as KRAS G12D/R/V/W, G13D, and Q61H, which reactivate KRAS; KRAS R68S, H95D/Q/R, and Y96C, which occur in the switch II pocket and hinder drug binding; and KRAS G12C amplification. ii) RAS/MAPK/PI3K alterations, including the activation of NRAS and PI3K; the activation of the downstream effector kinases BRAF, CRAF, and MEK1; and loss-of-function mutations in PTEN and NF1. iii) RTK alterations, including the activation of MET, ALK, RET, and FGFR3. iv) Tumor cells undergo histopathological changes, transitioning from lung adenocarcinoma to squamous lung cancer. Another study of 43 patients treated with sotorasib reported similar resistance mechanisms[114]. The sensitivity of different secondary genetic alterations to different KRAS G12Ci may vary, with certain

secondary mutations conferring resistance to both drugs (*e.g.*, Y96D and Y96S are resistant to either sotorasib or adagrasib), while others are resistant to only one drug[188,189]. The complexity of the problem is further exacerbated by the emergence of multiple resistance mechanisms in some patients and the presence of subclonal heterogeneity of resistance mechanisms within the tumor[114].

6.1.2. *Approaches to address KRAS G12Ci resistance*

To address the resistance mechanisms to KRAS G12Ci, various therapeutic strategies can be employed: i) KRAS G12Ci with novel mechanisms of action. An example is the Y96D mutation, which hinders adagrasib binding and consequently leads to resistance. However, the tri-complex active-state KRAS G12Ci RM-018 can still bind and inhibit KRAS G12C/Y96D mutant and overcome resistance[186]. ii) Pan-KRASi with broader targets[31]. Pan-KRASi can potentially reduce the KRAS-GTP burden by various KRAS mutants and may be effective against secondary KRAS mutations. iii) Targeting drug-modified targets. Zhang et al. reported an immune-based therapy based on neoepitopes generated by KRAS G12Ci (ARS1620) that may prove effective against drug resistance[190,191]. iv) Combination regimens with other drugs. Vertical combination strategies with other RAS/MAPK pathway inhibitors or horizontal combination strategies with other signaling pathway inhibitors can prolong resistance and achieve sustained inhibition. Preclinical evidence suggests that the resistance of KRAS G12Ci can be overcome by combining with EGFRi, SOS1i, SHP2i, type II RAFi, and MEKi[114,192,193]. A phase III clinical trial involving 160 patients with chemorefractory CRC reported that the combination of sotorasib and panitumumab (EGFRi) significantly extended progression-free survival (PFS) compared to the standard treatment regimen[194]. However, the overall response (OS) did not significantly improve with the combination regimen, possibly due to the limited duration of follow-up (median follow-up of 7.8 months). Another phase I clinical trial reported an ORR of 62.5% for the combination of divarasib plus cetuximab (EGFRi) in patients with metastatic CRC who had not previously received KRAS G12Ci ($n = 24$)[195], and the ORR of divarasib alone is 35.9%[64]. The median PFS for patients receiving the combination regimen was 8.1 months (95% CI: 5.5−12.3). Among the other five patients who had previously received KRAS G12Ci treatment, three achieved PR, and two had SD with the combination regimen. In addition to EGFRi, clinical trials combining SOS1i, SHP2i, mTORi, CDK4/6i, and anti-PD-1/PD-L1 immunotherapy with KRAS G12Ci are currently underway (NCT04330664, NCT04185883, and NCT03785249).

6.2. *Mechanisms of resistance to type II RAFi*

Extensive research has been conducted on the mechanisms of resistance to RAF monomer inhibitors, yet limited knowledge exists regarding resistance mechanisms specific to type II RAFi. ARAF mutations can cause compensatory activation of ERK signaling when BRAF and CRAF are inhibited, potentially acting as a shared resistance mechanism for type II RAFi[116]. Kelli et al. reported that ARAF played a significant role in conferring resistance to naporafenib[18]. Decreased ARAF expression may increase the susceptibility of RAS mutant cells to this medication. Resistance was linked to the kinase activity and dimerization properties of ARAF, resulting in paradoxical ERK activation in cells expressing only ARAF. Similarly, Ivana et al. discovered that acquired ARAF mutations (such as ARAF G387) decreased the flexibility of the hinge region and hindered the attachment of belvarafenib to RAF proteins. This resulted in resistance against belvarafenib in an ARAF kinase- and dimer-dependent manner in BRAF- and NRAS-mutated melanoma[116]. Despite resistance to other type II RAFi (naporafenib and AZ628), these ARAF mutations remained vulnerable to downstream MEKi and ERKi. The combination of belvarafenib with MEKi (cobimetinib) could overcome the resistance driven by ARAF mutations.

6.3. *Combination therapy based on type II RAFi in RAS mutant tumors*

The MAPK pathway relies predominantly on linear cascade phosphorylation for signal transduction. Still, its regulation is remarkably complex, involving multiple feedback loops at different cascade levels and interactions with other signaling pathways[196]. Therefore, combination approaches are considered a more promising strategy for treating RAS mutant tumors (Fig. 5). Both preclinical (Table 5) and clinical (Table 6) evidence have demonstrated that combination therapies based on type II RAFi show significant efficacy in treating RAS mutant tumors. In addition to vertical and horizontal combination strategies, synergistic effects of type II RAFi with several chemotherapeutic agents and anti-PD-1/PD-L1 immunotherapy have also been reported.

6.3.1. *Combined with other MAPK pathway inhibitors*

Multiple preclinical studies have substantiated the synergistic antitumor efficacy of type II RAFi when combined with MEKi (especially cobimetinib[116], trametinib[14], and mirdametinib[135,137]) in tumors harboring KRAS and NRAS mutations. Notably, type II RAFi exhibit more pronounced synergistic effects with MEKi than type I1/2 RAFi[5]. However, not all MEKi exhibit the same degree of synergy, which may be attributed to their impact on RAF–MEK complexes[5]. Combination therapy with type II RAFi and MEKi induces the BRAF/CRAF complex and prevents MEK from dissociating from the RAF complex. This reduces MEK dimerization and MEK-ERK interactions, leading to sustained inhibition of ERK activation[14].

Type II RAFi, in combination with MEKi or ERKi, has demonstrated significant clinical efficacy in treating RAS mutant tumors. In a phase Ib clinical trial, naporafenib plus trametinib (MEKi) displayed preliminary antitumor efficacy in melanoma patients with NRAS mutations, resulting in a DCR of 73% (22/30) and a PR rate of 30% (9/30) (NCT02974725)[177]. However, this combination therapy did not yield satisfactory results in KRAS mutant NSCLC, with only one PR observed out of 25 patients. Another phase II trial demonstrated the superiority of naporafenib plus trametinib in NRAS-mutated melanoma individuals who had experienced advancement after prior immunotherapy (NCT04417621). The DCR was 67% (16/24), and the PR rate was 4% (1/24) in this cohort[52]. For these patients, naporafenib plus LTT462 (ERKi) also exhibited strong efficacy (DCR of 62%). Furthermore, belvarafenib plus cobimetinib (MEKi) showed impressive antitumor activity against NRAS-mutated, immuno-resistant melanoma, resulting in a PR rate of 45% (5/11) (NCT03284502)[178,179]. In addition to its efficacy in melanoma, the combination of type II RAFi and MEKi also shows promise for treating other solid tumors. This is exemplified by the efficacy of lifirafenib plus mirdametinib in patients with RAS-mutated gynecologic tumors (NCT03905148)[51]. Clinical trials are ongoing to evaluate other combination regimens of type II RAFi plus MEKi to treat tumors with RAS mutations, including tovorafenib plus pimasertib

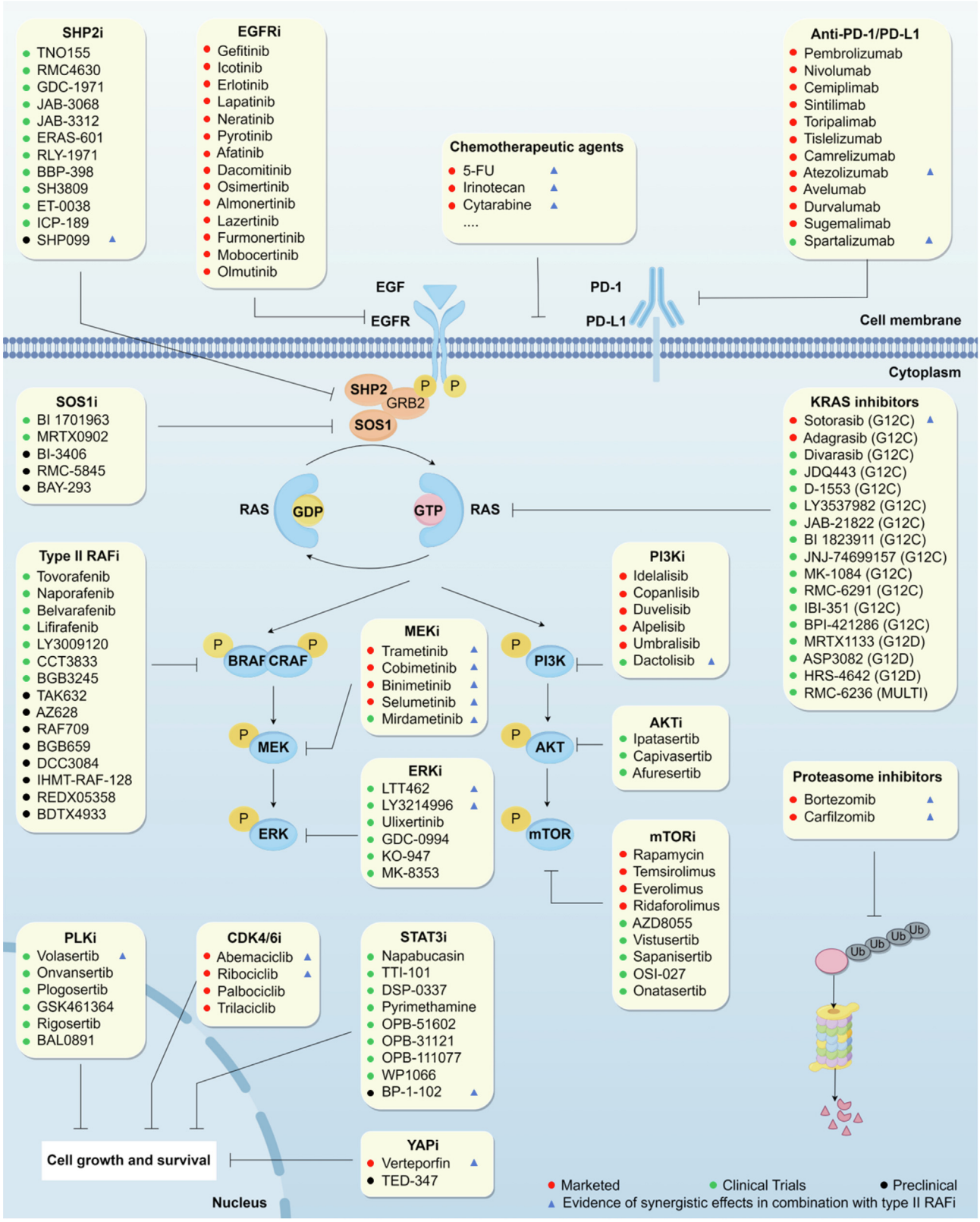

Figure 5 Combination regimens based on type II RAFi in RAS mutant tumors. Type II RAF inhibitors demonstrate synergistic antitumor activity when combined with other targeted agents, chemotherapeutic agents, or immunotherapies in RAS mutant tumors.

Table 5 Preclinical evidence of type II RAFi combination regimens in RAS mutant tumors.

Combination regimens	Synergistic effect	Ref.
Combined with MAPK pathway inhibitors		
Tovorafenib+TAK733 (MEKi)	RAS mutants in melanoma and CRC	197
Naporafenib+Trametinib (MEKi)	NRAS and HRAS mutants in bladder cancer	198
Belvarafenib+Cobimetinib (MEKi)	NRAS mutants in melanoma	116,182
Lifirafenib+Mirdametinib/ Selumetinib/Trametinib (MEKi)	KRAS and NRAS mutants in melanoma, CRC, and lung cancer	14,135, 137
LY3009120+Cobimetinib (MEKi)	NRAS mutants in melanoma; KRAS mutants in CRC and lung cancer	5,199
LY3009120+Trametinib (MEKi)/LY3214996 (ERKi)	NRAS and HRAS mutants in rhabdomyosarcoma	200
BGB3245+Mirdametinib (MEKi)	NRAS mutants in melanoma	137
TAK632+TAK733 (MEKi)	NRAS mutants in melanoma	129
AZ628+Cobimetinib/ Selumetinib (MEKi)	KRAS mutants in CRC and lung cancer	5,111
RAF709+Trametinib (MEKi)	KRAS mutants in CRC, lung, and pancreatic cancer	14,140, 201
DCC3084+Cobimetinib/ Binimetinib (MEKi)	KRAS mutants in lung and pancreatic cancer	131
Naporafenib+Sotorasib (KRAS G12Ci)	Lung cancer cells that are resistant to KRAS G12Ci	114
Naporafenib+SHP099 (KRAS SHP2i)	KRAS mutants in gastroesophageal adenocarcinomas	202
Combined with other signaling pathway inhibitors		
LY3009120+Abemaciclib (CDK4/6i)	NRAS mutants in melanoma; KRAS mutants in CRC and lung cancer	203
Naporafenib+Volasertib (PLK1i)	NRAS mutants in lung cancer	180
TAK632+Saracatinib/ Bosutinib (SRCi)	KRAS mutants in CRC, lung, and pancreatic cancer	136
AZ628+Dasatinib (SRCi)	KRAS mutants in breast cancer	204
AZ628+BP-1-102 (STAT3i)	KRAS mutants in lung cancer	205
AZ628+Dactolisib (PI3Ki/ mTORi)	KRAS mutants in CRC	206
LY3009120+Verteporfin (YAPi)	KRAS mutants in pancreatic cancer	207
Tovorafenib+Selinexor (CRM1i)	KRAS mutants in multiple myeloma	208
Tovorafenib+Bortezomib (proteasome inhibitor)	KRAS mutants in multiple myeloma	209
TAK632+Bortezomib/ Carfilzomib (proteasome inhibitor)	KRAS and NRAS mutants in multiple myeloma	210
Combined with chemotherapeutic agents		
LY3009120+Cytarabine	KRAS and NRAS mutants in AML	211,212
AZ628+5-FU+Irinotecan	KRAS mutants in CRC	213
Combined with immunotherapy		
Belvarafenib+Atezolizumab (anti-PD-L1)	NRAS mutants in melanoma	182

AML, acute myeloid leukemia; CRC, colorectal cancer.

(NCT04985604), belvarafenib plus cobimetinib (NCT04835805), and BGB3245 plus mirdametinib (NCT05580770).

The remarkable efficacy of type II RAFi, when combined with MEKi or ERKi, highlights the importance of achieving continuous and effective suppression of MAPK signaling to treat RAS-mutated tumors[135]. Hence, combining type II RAFi with other MAPK pathway inhibitors may also yield significant synergistic effects. An example of this is the frequent reactivation of RAS-GTP caused by negative feedback from ERK[5], and the combination of naporafenib plus either sotorasib (KRAS G12Ci) or SHP099 (SHP2i) showed synergistic effects on KRAS-mutated lung cancer and gastric cancer separately[114,202]. Based on these

Table 6 Clinical outcomes of type II RAFi combination regimens in RAS mutant tumors.

Phase	Trial number	Pts	Administration	Efficacy in RAS mutant tumors	Safety	Ref.
Ib	NCT02974725	66	Naporafenib+Trametinib (MEKi): 1. Naporafenib 200 mg BID+trametinib 1 mg QD; 2. Naporafenib 400 mg BID+trametinib 0.5 mg QD.	1. NRAS-mutant melanoma (n = 30): DCR: 73% (22/30); PR: 30% (9/30); SD: 43% (13/30); PD: 20% (6/30). 2. KRAS-mutant NSCLC (n = 25): PR: 4% (1/25).	1. Well tolerance; 2. DLTs were observed; 3. TEAEs grade ≥3: 77% (23/30). Rash: 23% (7/30); Anamia: 13% (4/30).	177
II	NCT04417621 (Abstract)	134	Naporafenib+LTT462 (ERKi): Naporafenib 400 mg BID +LTT462 200 mg QD. Naporafenib+Trametinib (MEKi): Naporafenib 200 mg BID +trametinib 1 mg QD. Naporafenib+Ribociclib (CDK4/6i): Naporafenib 400 mg BID +ribociclib 400 mg QD.	NRAS-mutant, immunoresistant melanoma (n = 29): DCR: 62% (18/29); PR: 21% (6/29); SD: 41% (12/29); PD: 31% (9/29). NRAS-mutant, immunoresistant melanoma (n = 24): DCR: 67% (16/24); PR: 4% (1/24); SD: 63% (15/24); PD: 17% (4/24). NRAS-mutant, immunoresistant melanoma (n = 15): DCR: 33% (5/15); PR: 7% (1/15); SD: 27% (4/15); PD: 40% (6/15).	1. Well tolerance; 2. TEAEs grade ≥3: 46% (62/134). Rash: 7% (9/134).	52
Ib	NCT03284502 (Abstract)	118	Belvarafenib + Cobimetinib (MEKi): 1. Belvarafenib 200 mg BID+cobimetinib 20 mg QD; 2. Belvarafenib 300 mg BID+cobimetinib 20 mg QOD.	1. NRAS-mutant melanoma (n = 19): PR: 26% (5/19); SD: 42% (8/19). 2. 2 pts with KRAS G13-mutant CRC and 1 pt with HRAS-mutant melanoma showed PR.	1. Well tolerance; 2. DLTs were observed; 3. TEAEs: Dermatitis acneiform: 53% (62/118); Diarrhea: 28% (33/118); Rash: 27% (32/118).	178, 179
Ib	NCT03905148 (Abstract)	56	Lifirafenib + Mirdametinib (MEKi): Lifirafenib 15−20 mg QD +mirdametinib 2−4 mg QD or BID.	1. LGSOC with MAPK pathway aberrations (RAS mutation rate is not available, n = 17): DCR: 94% (16/17); CR: 6% (1/17); PR: 53% (9/17); SD: 35% (6/17); PD: 6% (1/17). 2. 1 pt with NRAS Q61K-mutant NSCLC, 1 pt with KRAS G12A-mutant endometrial cancer, and 1 pt with KRAS G12V-mutant low-grade serous adenocarcinoma of Mullerian origin showed PR.	1. Well tolerance; 2. DLTs were observed; 3. Grade 3 TEAEs: 43% (24/56).	51
I	NCT02607813	43	Naporafenib + PDR001 (anti-PD-1): Naporafenib 400 mg BID + PDR001 400 mg Q4W.	1. NRAS-mutant melanoma (n = 21): DCR: 52% (11/21); CR: 5% (1/	1. Well tolerance; 2. NO DLTs were observed;	176

Table 6 (*continued*)

Phase	Trial number	Pts	Administration	Efficacy in RAS mutant tumors	Safety	Ref.
				21); PR: 0% (0/21); SD: 48% (10/21); PD: 24% (5/21). 2. KRAS-mutant NSCLC (n = 22): DCR: 46% (10/22); PR: 14% (3/22); SD: 32% (7/22); PD: 32% (7/22).	3. TEAEs grade ≥3: 49% (21/43). Rash: 14% (6/43).	

MEKi, MEK inhibitor (same with other inhibitors); QD, once daily; BID, twice a day; QOD, three times a week; Q4W, every four weeks; DCR, disease control rate; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NSCLC, non-small cell lung cancer; CRC, colorectal cancer; LGSOC, low-grade serous ovarian carcinoma; DLT, dose-limiting toxicity; TEAEs, treatment-emergent adverse events; pts, patients.

findings, it can be inferred that type II RAFi may also have synergistic antitumor effects when combined with drugs such as SOS1i or EGFRi.

6.3.2. *Combined with inhibitors targeting other signaling pathways*

The complex interaction between the MAPK pathway and other pathways greatly influences the emergence of innate or acquired resistance in RAS-mutated tumors. Some RAS mutant cells exhibit reduced sensitivity to type II RAFi, in which the RAS mutation does not serve as the primary oncogenic driver gene[214]. Consequently, combining type II RAFi with inhibitors targeting the crosstalk pathways is an attractive approach.

Specifically, the combination of type II RAFi with inhibitors of the cell cycle pathway (CDK4/6i and PLK1i) exhibits synergistic effects. The combination of LY3009120 plus abemaciclib (CDK4/6i) resulted in increased G0/G1 arrest in tumor cells with KRAS and NRAS mutations, mainly due to increased suppression of phospho-Rb and cyclin D1[203]. A phase II trial showed the synergistic effect of combining naporafenib and ribociclib (CDK4/6i) in immune-resistant melanoma patients with NRAS mutations (NCT04417621)[52]. The study showed moderate efficacy, with a DCR of 33% (5/15) and a PR of 7% (1/15). However, this combination's efficacy was weaker than that of naporafenib in combination with MEKi or ERKi therapies[52]. PLK1 is another prominent cell cycle regulator and interacts with CRAF, promoting ERK activation and tumor progression[215]. Both *in vitro* and *in vivo* studies have demonstrated that coadministration of PLK1i has synergistic effects on KRAS-mutated tumors[216]. Notably, PLK1i exhibit heightened efficacy in treating KRAS mutant CRC[217,218], and ongoing clinical studies are investigating the potential therapeutic benefits (NCT03829410). In NSCLC cells harboring NRAS mutations, the combination of naporafenib and volasertib (PLK1i) was found to be lethal, leading to the arrest of the cell cycle at the G2/M phase[180].

Previous studies have shown that simultaneous targeting of the SRC/STAT3 (SRCi, STAT3i) pathway in combination with type II RAFi has synergistic antitumor effects. Specifically, the combination of AZ628 and dasatinib (SRCi) successfully inhibited the growth of KRAS-mutated breast cancer cells[204]. The synergistic effects of AZ628 and BP-1-102 (STAT3i) were observed in lung cancer cells with KRAS mutations by abrogating the MAPK pathway[205]. Moreover, CCT3833 targets both pan-RAF and SRC. Although the exact mechanism of action of CCT3833 on RAF dimers has not been determined, docking studies have proposed that CCT3833 acts as a type II RAFi[136]. Preclinical evidence has shown that CCT3833 exhibits significant activity in KRAS-mutated CRC, pancreatic cancer, and lung cancer[136]. CCT3833 showed a favorable effect on one patient diagnosed with spindle cell sarcoma, who had the KRAS G12V mutation and did not respond to other inhibitors[136]. The activation of the SRC/STAT3 pathway has a complex interaction with RAS-MAPK signaling that needs to be further elucidated[219]. These results offer encouraging preclinical evidence for combining RAF and SRC inhibition to treat tumors with RAS mutations.

Moreover, blocking the MAPK pathway has been discovered to significantly trigger the PI3K/AKT/mTOR pathway[220]. Consequently, AZ628 plus dactolisib (PI3Ki/mTORi) demonstrated synergistic effects on CRC cells with KRAS mutations[206]. In KRAS mutant pancreatic cancer, verteporfin (YAPi) could enhance LY3009120 by inhibiting AKT compensatory activation[207]. Additionally, proteasome inhibitors, such as bortezomib and carfilzomib, are vital clinical significance in treating multiple myeloma[221]. The activation of RAS or RAF mutations increases proteasomal activity, leading to resistance against proteasome inhibitors[210]. Notably, the combination of type II RAFi with proteasome inhibitors exhibited a synergistic inhibitory effect in multiple myeloma with RAS mutations, such as tovorafenib plus bortezomib and TAK632 plus carfilzomib[209,210].

6.3.3. *Combined with chemotherapeutic agents*

Type II RAFi could enhance the efficacy of several DNA-damaging chemotherapeutic agents and synergistically inhibit tumor growth. Carlota et al. reported that IKKα (p45) was required for efficient DNA repair. DNA damage rapidly activates IKKα (p45), dependent on MAPK signaling[213]. Based on this, researchers proposed that RAF inhibition could synergistically enhance the efficacy of DNA-damaging chemotherapeutic agents. In patient-derived KRAS mutant CRC tumoroids, AZ628 showed improved therapeutic potential when combined with 5-FU and irinotecan, particularly in tumors resistant to chemotherapy[213]. Similarly, in RAS mutant acute myeloid leukemia cells, LY3009120 showed increased potency in combination with cytarabine, which inhibited cell proliferation mainly by disrupting DNA synthesis[211]. However, a clinical trial evaluating the regimen of tovorafenib plus paclitaxel showed insufficient efficacy and

disease progression in patients with KRAS- or BRAF-mutated NSCLC (NCT02327169). The distinct mechanism of action of paclitaxel might cause this lack of synergistic effects. Paclitaxel can bind to microtubule proteins to inhibit cell division rather than directly inducing DNA damage[222].

6.3.4. *Combined with immunotherapy*

Multiple inhibitors targeting RAS signaling could modulate the tumor immune microenvironment[4]. Type II RAFi have exhibited the potential to improve the efficacy of anti-PD-1/L1 immunotherapy. Yu et al. discovered that belvarafenib plus atezolizumab (anti-PD-L1) showed more significant inhibitory activity against NRAS mutant melanoma in mice than single drug treatment[182]. The combined treatment significantly boosted the infiltration of $CD8^+$ T cells, and the improved inhibition of tumor growth was strongly associated with the increase in $CD8^+$ T cell levels within the tumor microenvironment. A clinical trial involving 43 patients has provided additional evidence supporting the potential synergistic effects of type II RAFi in combination with anti-PD-1 immunotherapy (NCT02607813)[176]. Among patients with NRAS-mutated melanoma, the combination of naporafenib and PDR001 (anti-PD-1) demonstrated a DCR of 52% (11/21), with one patient achieving a complete response (CR). In patients with KRAS-mutated NSCLC, the regimen resulted in a DCR of 46% (10/22), with three patients achieving PR. Unfortunately, another clinical trial investigating tovorafenib in combination with nivolumab was prematurely terminated (NCT02723006). Moreover, the efficacy of combining type II RAFi with MEKi in RAS mutant tumors was found to rely on the presence of $CD8^+$ T cells[14]. The presence of T cells within the tumor was reduced by MEKi (trametinib), but this effect was reversed when combined with type II RAFi (BGB283). This combination increased the proportion of $CD8^+$ T cells and caused long-lasting immune infiltration. $CD8^+$ T cells were required for tumor continued inhibition, while anti-PD-L1 therapy activated these cells and showed increased efficacy[14]. Consequently, this investigation offers a rationale for conducting triple therapy trials involving type II RAFi, MEKi, and anti-PD-1/L1 therapy. Currently, an ongoing phase I clinical trial is evaluating the efficacy of belvarafenib plus cobimetinib (MEKi) plus nivolumab (anti-PD-1) in advanced melanoma patients with NRAS mutations who previously received anti-PD-1/L1 therapy (NCT04835805). Additional research is necessary to gain a complete understanding of the exact underlying mechanisms involved. However, the utilization of type II RAFi plus anti-PD-1/L1 in a dual regimen, or with the addition of MEKi in a triple regimen, can show potential in treating RAS-mutated tumors.

7. Summary and prospects

Although significant breakthroughs have been made in targeting KRAS G12C, directly targeting RAS remains challenging given the structural specificity and diverse biological functions, particularly in non-G12C RAS mutant tumors. Therefore, a promising research strategy is inhibiting crucial downstream kinases of RAS, particularly RAF. However, there is currently limited knowledge about the role of different RAF homo and heterodimers in various tumors with different RAS mutations. Nonetheless, the notion that RAF dimerization is vital for the emergence of drug resistance *via* ERK reactivation is now well accepted. To address the issue of RAF dimerization, new-generation RAFi employ strategies such as inhibiting RAF dimer activity, disrupting RAF dimer formation, and targeting DIF.

Currently available inhibitors targeting RAF dimers exhibit various effects on different types of RAF monomers and dimers, some of which may result in drug insensitivity or resistance. Complete suppression of MAPK signaling is necessary to achieve significant antitumor effects in RAS mutant tumors[6]. Additionally, the crosstalk between MAPK signaling pathway and other signaling pathways is complex. Thus, targeting specific RAS mutations and the RAF dimer responsible for driving ERK signaling is imperative[7]. It is crucial to use drugs with well-defined actions and mechanisms to ensure their effectiveness, as failure to do so may result in paradoxical ERK activation.

Previous studies on RAF dimers have focused mainly on BRAF and CRAF dimers due to their high kinase activity and dimerization capacity. However, emerging studies indicate the significance of ARAF dimers in drug resistance mechanisms, although ARAF exhibits a decreased dimer formation ability. The impact of most inhibitors targeting RAF dimers on ARAF monomers and dimers is unknown. Based on the existing data, it is plausible that most type II RAFi may exhibit inadequate inhibition of ARAF[150], and further investigations are imperative to validate this hypothesis. The underlying structural and mechanistic rationale behind the ineffective inhibition of ARAF requires further exploration. Therapeutically, the lack of ARAF inhibition can have positive and negative implications[18]. On the one hand, potential outcomes may include decreased effectiveness and the emergence of drug resistance. On the other hand, sparing ARAF could potentially widen the therapeutic window since patients may be intolerant to completely inhibiting the MAPK pathway[150].

Current inhibitors targeting RAF dimers have shown varying efficacy in different tumor types. Paradox breakers have limited efficacy in treating RAS mutant tumors, while type II RAFi have demonstrated significant therapeutic benefits in patients with NRAS- and KRAS-mutated tumors, particularly in melanoma. Notably, type II RAFi, such as belvarafenib, tovorafenib, and the recently published DCC3084 and BDTX4933, exhibit promising potential for penetrating the blood−brain barrier[131,133]. This heightened permeability of type II RAFi may offer clinical advantages for patients with brain metastases and CNS tumors compared to first-generation RAFi. However, the effectiveness of type II RAFi monotherapy is limited in individuals with CRC and NSCLC. The reasons for variations in drug sensitivity across different tumor types remain to be thoroughly investigated. The efficacy of type II RAFi monotherapy in RAS mutant tumors should be viewed cautiously due to the sparing of ARAF, and single-target inhibitors typically reactivate MAPK signaling.

The mechanisms underlying resistance to type II RAFi are poorly understood, although recent evidence suggests a potential association with reactivation of ARAF dimer-induced ERK signaling. It is crucial to investigate the effectiveness and resistance mechanisms of type II RAFi in RAS mutant tumors, particularly the combination therapies targeting the identified resistance mechanisms. Ongoing clinical trials have shown exciting results in combining type II RAFi with MEKi or ERKi for managing NRAS and KRAS mutant tumors. This approach has shown promising efficacy in patients with melanomas who have received prior immunotherapy. Due to the critical role of RAF dimerization in ERK signaling reactivation, which often leads to drug resistance, type II RAFi can potentially address the issue of drug resistance in other MAPK pathway inhibitors. These clinical

findings suggest that type II RAFi may be a promising approach for clinical application against RAS mutant tumors. In addition to combination with MAPK pathway inhibitors, other combination regimens involving type II RAFi have also shown promise in RAS mutant tumor cells. These include combinations with inhibitors targeting other pathways, DNA-damaging chemotherapeutic agents, and anti-PD-1/L1 immunotherapy, although further validation through clinical trials is needed to confirm their effectiveness. Thus, targeting RAF dimers is expected to be further studied and developed for treating RAS mutant tumors.

Nevertheless, combination therapies often result in increased toxicity, potentially limiting their clinical utility. While achieving significant efficacy is important, another crucial goal is minimizing combination therapies' effects on normal cells to improve patient tolerability. In addition to developing and optimizing combination regimens that target specific RAS mutant tumors, improving drug targeting strategies represents a viable approach. One prevalent issue is the inadequate localization of small-molecule kinase inhibitors to tumors due to oral administration. Interactions with diverse food substances can affect the plasma concentration of drugs[223]. Thus, the different pharmacokinetics and distributions within the body hinder the optimal synergistic effect of coadministration. To address this issue, exploring novel strategies, such as utilizing nanomaterials and other tumor-targeted co-delivery systems, may offer promising prospects[224,225]. These systems could enable the precise administration and controlled release of drugs within the body, thereby ensuring continuous suppression of tumor cell growth, achieving the best therapeutic effect at the smallest dose, and reducing the adverse effects of combined treatments.

Declaration of Generative AI and AI-assisted technologies in the writing process

While preparing this paper, the authors used Home for Researchers (www.home-for-researchers.com) to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and took full responsibility for the publication's content.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (82073059, 82073314), the Sichuan Science and Technology Program (2023NSFSC1838, China), and the Sichuan University Postdoctoral Interdisciplinary Innovation Fund (JCXK2212, China).

Author contributions

Hongwei Xia and Feng Bi conceived and supervised the work. Huanhuan Yin summarized the literature, drafted the manuscript, and plotted the tables and figures. Qiulin Tang proofread the tables and figures. Hongwei Xia and Feng Bi revised and edited the manuscript. All authors approved the final manuscript.

Conflicts of interest

All authors declare no conflicts of interest.

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