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REVIEW ARTICLE

Targeting RAS−RAF−MEK−ERK signaling pathway in human cancer: Current status in clinical trials

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Abstract Molecular target inhibitors have been regularly approved by Food and Drug Administration (FDA) for tumor treatment, and most of them intervene in tumor cell proliferation and metabolism. The RAS−RAF−MEK−ERK pathway is a conserved signaling pathway that plays vital roles in cell proliferation, survival, and differentiation. The aberrant activation of the RAS−RAF−MEK−ERK signaling pathway induces tumors. About 33% of tumors harbor RAS mutations, while 8% of tumors are driven by RAF mutations. Great efforts have been dedicated to targeting the signaling pathway for cancer treatment in the past decades. In this review, we summarized the development of inhibitors targeting the RAS−RAF−MEK−ERK pathway with an emphasis on those used in clinical treatment. Moreover, we discussed the potential combinations of inhibitors that target the RAS−RAF−MEK−ERK signaling pathway and other signaling pathways. The inhibitors targeting the RAS−RAF−MEK−ERK pathway have essentially modified the therapeutic strategy against various cancers and deserve more attention in the current cancer research and treatment.

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  Peer review under responsibility of Chongqing Medical University.
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Introduction

Molecular targeted therapy has been considered a valuable therapy in tumor treatment because of its small size, selective binding with a wide range of extracellular and intracellular targets, superiority over cytotoxic chemotherapy, and few side effects.[1] In the last 20 years, 43 molecule target drugs have been approved by the Food and Drug Administration in the US.[2] Most of these inhibitors target the kinases that regulate the cell proliferation, metabolism, and immune modulation.

RAS—RAF—MEK—ERK is a classical pathway from the 1990s and transmits extracellular signals to specific intracellular targets (Fig. 1, created with BioRender.com).[3] A vast map of substrates related to cell proliferation, differentiation, and metabolism are regulated by ERK.[4–6] Statistically, 33% of RAS mutations and 8% of BRAF mutations are observed in all human cancers thus far.[7] The aberrant activation of the signaling pathway contributes to tumorigenesis and tumor development.[8–10] To this end, many inhibitors targeting this pathway are developed, which has essentially modified the therapeutic strategy of cancers. Potential combinations are explored in preclinical and clinical studies. In this review, we provide an overview of the current literature on the cascade activation of RAS—RAF—MEK—ERK and the development of inhibitors targeting this pathway. We hope this will throw light on the design of better therapeutic strategies.

Components and activation of RAS—RAF—MEK—ERK signaling pathway

Important upstream molecular marker-RAS

As an important upstream molecular marker of the RAS—RAF—MEK—ERK pathway, RAS serves as a molecular switch by binding GTP/GDP,[11] and it includes four isoforms: HRAS, KRAS4A, KRAS4B, and NRAS.[12] KRAS is the most frequent isoform in all human cancers.[13] KRAS4A and KRAS4B are the different splicing isoforms of the same gene. When the transmembrane receptors (receptor tyrosine kinase, RTKs) are activated, the complexes of growth-factor-receptor bound protein 2 (GRB2) and son of sevenless (SOS) in the cytoplasm are recruited to the inner surface of cell membrane.[14] SOS is an important guanine nucleotide exchange factor (GEF) that delivers the signal from RTK to RAS.[15] Then RAS—GDP turns into RAS—GTP with the help of SOS. In this process, GRB2 acts as a bridge between RTK and SOS. After that, the intrinsic RAS-GTPase hydrolyzes RAS—GTP to inactivated RAS—GDP with the help of GTPase-activating proteins (GAPs).[16]

The downstream kinase of RAS—RAF

RAF, the downstream kinase of RAS, is recruited and directly phosphorylated after RAS turns on the switch. It includes three isoforms: ARAF, BRAF, and CRAF.[17,18] All RAF isoforms share three conserved regions: conserved region 1 (CR1) containing RAS binding domain (RBD), conserved region 2 (CR2), which is the regulatory domain, and conserved region 3 (CR3), which is the kinase domain and contributes to the variable kinase activities (BRAF > CRAF > ARAF).[19,20] Except for three catalytically competent enzymes, the RAF family also has two pseudokinases, a kinase suppressor of RAS 1/2 (KSR1/2), which generally works as a scaffold in the RAS/MAPK pathway for bridging MEK to RAF.[21] RAF dimerization is widely considered as a key event in RAF activation. Active RAS induces hetero-dimerization of CRAF and BRAF, an effect that is dependent on the serine residue at position 621 of CRAF. In depth, RAS induces the CRAF—BRAF complex formation through the exposure of 14-3-3 binding sites in the COOH-terminus of CRAF.[22] The subsequent research reveals that several mutants of BRAF with reduced kinase activity activate MEK by activating the CRAF.[23] Wild-type B-RAF forms a complex with C-RAF in a RAS-dependent
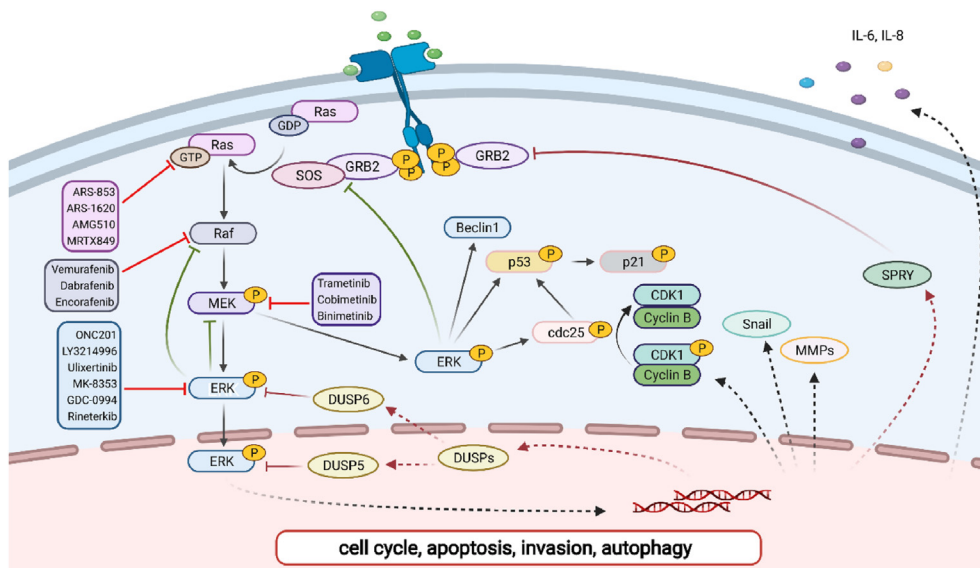

Figure 1 Signal transduction of RAS—RAF—MEK—ERK pathway.

manner, while the mutants bind independently of RAS. Importantly, wild-type B-RAF can also activate C-RAF through a mechanism involving 14-3-3 mediated hetero-oligomerization and C-RAF transphosphorylation.[24] Except for the kinase domain, the constitutive phosphorylation in N-terminal of BRAF also activates the CRAF while N-terminal of CRAF is dependent on MEK, suggesting a feedback mechanism and explaining a key difference between BRAF and CRAF. In addition to the hetero-dimerization of CRAF and BRAF, RAF-related pseudo-kinase KSR also hetero-dimerizes with RAF, thereby triggering RAF activation.[25]

Downstream molecular-MEK and ERK

MEK1 and MEK2, downstream kinase of RAF, are dual-specific kinase that catalyzes both tyrosine and serine/threonine residues in ERK1 and ERK2.[26] The mutations of MEK that enhance MEK1 homodimerization promotes the activation of RAF kinase and catalytic activity toward the kinase ERK.[27] Unlike the narrow map of substrates for MEKs, ERKs phosphorylate hundreds of substrates, including transcriptional factors and protein kinases.[28] ERKs work both in the cytoplasm (BIM, MCL, RSK, and MNK) and the nucleus (CREB, FOS, MYC, JUN, MSK, ELK, and ETS).[12]

Feedback regulation

RAS—RAF—MEK—ERK cascade is regulated by the negative feedback, which is generally divided into short term and long term. The short-term feedback of the ERK cascade includes the direct inhibition of RAS, RAF, and MEK1 through ERK1/2.[29—34] In detail, activated ERK breaks the interaction of RAF—RAS by phosphorylating SOS, and phosphorylates the Ser/Thr sites of BRAF, thereby inhibiting the binding to the activated RAS and disrupting heterodimerization with CRAF.[31] Additionally, the N-terminus of MEK is also directly phosphorylated by the activated ERK. The long-term feedback is the activation of transcriptional factors (DUSPs and SPRYs). Dual specificity protein phosphatases (DUSPs), a kind of phosphatase for ERK, are spatially located. The DUSP6 works in the cytoplasm while DUSP5 in the nucleus.[35] Another one is that sprouty proteins (SPRYs) bind with Grb2 and inhibit the recruitment of the Grb2—SOS complex. In addition, SPRYs can also down-regulate the RAS—GTP by forming the juxta-cytoplasmic membrane localization of NF1.[35,36]

Crosstalk with PI3K pathway

The RAS—RAF—MEK—ERK pathway is also regulated by the bypath, RAS—PI3K—AKT—mTOR. Activated RAS recruits PI3K via GAB binding proteins. Subsequently, PIP3 generated through PI3K modification activates the AKT and mTOR which regulates cell growth, survival, and metabolism.[37] The crosstalk between RAS/RAF/ERK and RAS/PI3K/AKT/mTOR can be summarized as cross-inhibition and pathway convergence on substrates. Cross-inhibition refers to that a member of one way negatively regulates an upstream component of another way. For example, active ERK blocks the insulin by phosphorylating GAB1 at the level of PI3K, and AKT negatively regulates ERK activation by

phosphorylating RAF at Ser259.[38,39] The latter crosstalk, i.e., pathway convergence including FOXO, C-MYC, BAD and GSK3, shares the same substrates activated by those signal pathways and promotes the cell survival, proliferation and metabolism.[40,41]

Inhibitors of RAS—RAF—MEK—ERK pathway

RAS inhibitors

The frequent mutations of RAS—RAF—MEK—ERK pathway are observed in human cancers, with 33% RAS mutations and 8% BRAF mutations, and the hyperactivity of RAS—RAF—MEK—ERK is highly related to tumor progression.[7] Targeting the RAS—RAF—MEK—ERK pathway represents an attractive method for alleviating the tumor growth. At present, many inhibitors have been developed, and the discussed inhibitors are listed in the Table 1. The treatment of RAS mutant cancers is unsatisfactory because of the lack of efficient and specific targets. A targetable RAS mutation, $KRAS^{G12C}$, has been observed in 14% of lung cancers and 5% of colorectal cancers.[95,96] Schokat group firstly reported a compound, i.e., compound 12, that could irreversibly bind to the cysteine of $KRAS^{G12C}$ instead of glycine of $KRAS^{WT}$.[97] Subsequently, a series of inhibitors based on the structural optimization and transformation of compound 12 are developed, such as ARS-853 (the first $KRAS^{G12C}$-specific compound with cellular activities, 2013) and ARS-1620 (the first $KRAS^{G12C}$-specific compound with *in vivo* activity, 2018).[98,99] The $KRAS^{G12C}$-specific drug, AMG510 (storasib), firstly went into clinical trial in 2019 and was subsequently proved by FDA in 2021.[42,43] Another $KRAS^{G12C}$-specific covalent inhibitor, MRTX849 (adagrasib) developed by Mirati group, also went into clinical trial in 2019.[44] However, the resistance to AMG510 and MRTX849 was observed recently, resulting in a lack of efficacy, recurrence, or progression.[45] At the same time, other strategies such as the destruction of RAS/effector interaction and the inhibition of RAS downstream molecules are also undergone vigorously. Therefore, we summarized the inhibitors targeting RAS downstream molecules following.

RAF inhibitors

Compared with CRAF and ARAF, BARF has a higher mutation frequency. The mutation $BRAF^{V600E}$ (also named $BRAF^{V599E}$, with a substitution of valine by glutamic acid at position 599) accounts for 95% of all BRAF mutations.[46,47,100] This mutation shows an elevated kinase activity by stabilizing the catalytically preferred conformation. PLX4720 is the first generation $BRAF^{V600E}$ inhibitor that targets the active DFG motif in ATP binding pocket of $BRAF^{V600E}$, which opens the door to target $BRAF^{V600E}$.[101] Vemurafenib (PLX4032) was subsequently derived and was approved by the United States and the European Union for the treatment of $BRAF^{V600}$-mutant melanoma in 2011 and 2012, respectively.[48,49,102] In clinical trials, vemurafenib suppressed the melanoma growth in a large proportion of patients, but ineffectiveness was observed after 7—8 months of treatment. Dabrafenib was another selective BRAF inhibitor after vemurafenib, and was approved for the treatment of

Table 1 Functional difference and FDA approved treatment of discussed inhibitors.

Inhibitors	Specific target	Mechanism	FDA approved treatment	Reference
ARS-853	KRAS^{G12C}	Covalent inhibitor	—	[42]
ARS-1620	KRAS^{G12C}	Covalent inhibitor	—	[43]
AMG510	KRAS^{G12C}	Covalent inhibitor	Locally advanced or metastatic NSCLC with KRAS^{G12C} mutation	[44—46]
MRTX849	KRAS^{G12C}	Covalent inhibitor	KRAS^{G12C}-mutated NSCLC	[47]
PLX4720	BRAF^{V600E}	An ATP-competitive RAF kinase inhibitor	—	[48]
Vemurafenib	BRAF^{V600E}	An ATP-competitive RAF kinase inhibitor	Metastatic melanoma with BRAF^{V600E} mutation, Erdheim—Chester Disease with BRAF^{V600} mutation	[49—51]
Dabrafenib	BRAF$^{V600E/K/D}$	An ATP-competitive RAF kinase inhibitor	Unresectable or metastatic melanoma with BRAF^{V600E} mutation	[52—59]
Encorafenib	BRAF$^{V600E/K}$	An ATP-competitive RAF kinase inhibitor	Unresectable or metastatic melanoma with BRAF$^{V600E/K}$ mutation	[60]
Trametinib	MEK1/2	Allosteric non-ATP competitive inhibitor	Unresectable or metastatic melanoma with BRAF$^{V600E/K}$, BRAF positive anaplastic thyroid cancer, Metastatic NSCLC with BRAF^{V600E} mutation	[61,62]
Binimetinib	MEK1/2	Allosteric inhibitor	Unresectable or metastatic melanoma with BRAF$^{V600E/K}$ or NRAS mutation	[63,64]
Cobimetinib	MEK1/2	Allosteric inhibitor	Advanced melanoma with BRAF$^{V600E/K}$ mutation	[65—71]
ONC201	AKT/ERK	—	Small cell lung cancer	[72—75]
LY3214996	ERK1/2	Reversible ATP-competitive kinase inhibitor	—	[76,77]
Ulixertinib	ERK1/2	Reversible ATP-competitive kinase inhibitor	—	[78—82]
MK-8353	ERK1/2	Reversible ATP-competitive kinase inhibitor	—	[83—87]
GDC-0994	ERK1/2	Reversible ATP-competitive kinase inhibitor	—	[88—91]
Rineterkib	RAF/ERK1/2	—	—	[92—94]

BRAF^{V600}-mutant melanoma in 2013.[50] Compared with vemurafenib, dabrafenib not only inhibits BRAF^{V600E}-mutant tumors, but also inhibits BRAF^{V600K}- and BRAF^{V600D}-mutant tumor cells, and this inhibitor particularly shows good efficacy for those tumors with BRAF^{V600E} mutation, such as melanoma, lung cancer, biliary tract cancer and thyroid cancer.[51—57] Nevertheless, the resistance to dabrafenib occurs after several months of treatment as well.[58] Encorafenib is a recently approved BRAF inhibitor, and shows a longer half-time of dissociation with lower abnormal activation of the MAPK pathway.[59] However, none of these inhibitors show significant benefits for KRAS-driven cancer patients, and all acquire resistance after long-term treatment.

MEK inhibitors

Although the activated mutations in MEK are relatively low in human tumors, the activity of MEK is found at high levels in more than 85% of cancers partly because of the upstream mutations of RAS or RAF. Therefore, inhibiting MEK is an attractive and novel therapeutic strategy. To this end,

several combinations (MEK inhibitor and BRAF inhibitor) were approved by FDA, and those therapeutic approaches effectively halted tumor growth in preclinical models and the patients with RAS or RAF mutations.[60,103] Trametinib is a non-ATP competitive inhibitor of MEK1 and MEK2 (IC$_{50}$ of 0.7 nM and 0.9 nM, respectively), and was approved by the FDA in 2013 for the treatment of unresectable melanoma with BRAF$^{V600E/K}$ mutation.[104] In 2017, the combination therapy of dabrafenib and trametinib was also approved by the FDA for the treatment of non-small cell lung cancer (NSCLC) with BRAF^{V600E} mutation.[105] Cobimetinib (GDC-0973) is an effective and highly selective allosteric MEK1/2 inhibitor.[106] This drug showed a good efficacy in BRAF and KRAS mutant cell lines and in BRAF$^{V600E/K}$-mutant patients.[107—112] Cobimetinib and vemurafenib were approved by the FDA and EMA (European Medical Agency) in 2015 for the treatment of BRAF$^{V600E/K}$ mutant and unresectable melanoma.[113] Binimetinib (MEK162 or ARRY-162 or ARRY-438162) is an effective and oral MEK1/2 inhibitor (IC$_{50}$ of 12 nM).[114] The combined treatment of binimetinib and encorafenib was approved by the FDA in 2018 for those patients with BRAF$^{V600E/K}$-mutant and unresectable

melanoma. Except for tumor inhibition, the side effects of combination therapy, especially the hyperkeratosis and verrucous keratosis, is significantly lower than that of monotherapy.[115] The high incidence of cutaneous side effects can be explained by the hyper-proliferation of epidermal keratinocyte, which is induced by the paradoxical activation of the MAPK pathway.[61] The addition of a MEK inhibitor alleviated the side effects by inhibiting the reactivated MAPK. However, resistance also occurs in patients after a period of treatment with BRAF/MEK combination therapy.[62]

Mechanisms of resistance to RAF and MEK inhibitors

Drug resistances markedly block the treatment efficacy of various human cancers thus far, including the targeted therapy. The approved RAFi/MEKi described above show advantages of high selectivity, initially effective inhibition, and low side effects; however, clinical responses to those inhibitors are highly variable among patients, and resistance occurs in less than one year without other intervention treatment.[65] The mechanisms of RAFi/MEKi resistance might be classified as intrinsic resistance or acquired resistance as reported.[66,67] The intrinsic, i.e. innate, means that the tumors do not respond to the drug treatment; the latter, i.e., adaptive, means that the responsive tumors develop the resistance under the stress of drug treatment. However, the two forms are similar to some degree, and the main mechanisms are discussed below.

Approaches to targeting KRAS-mutant or BRAF-mutant cancers have failed, often due to the upregulation of RTK signaling. For example, an *in vitro* study showed that the hepatocyte growth factor (HGF) secreted by stromal cells could induce the reactivation of ERK pathways via MET receptor, therefore mediating the resistance to vemurafenib-induced RAF inhibition in BRAF melanomas.[66] EGFR-mediated resistance to vemurafenib was observed in BRAF-mutant colorectal cancers and melanomas as well.[68,69] In addition, the RTKs also contribute to the MEKi resistance in KRAS-mutant cancers by ERK reactivation.[63,70,71,116] Therefore, blocking the signals from those activated RTKs might be a viable therapeutic approach. A recent study revealed that combining SHP099 (an inhibitor targeting SHP2 which acts between RTKs and RAS) and MEKi could re-sensitize both KRAS-mutant and wild-type KRAS tumors.[64] Notably, the RAF activation is regulated by RAF dimerization upon RAS activation in wild-type BRAF cells, while the mutant RAF catalytic function is regulated by its monomer.[25,117] Therefore, clinically used ATP-competitive inhibitors could cause "theoretical resistances" by stabilizing a rigid closed conformation of the kinase domain of RAF, thus promoting RAF dimerization and leading to hyperactivation of ERK pathways.[118] This intrinsic resistance was observed in wild-type BRAF tumors when treated with non-saturating concentrations of ATP-competitive inhibitors.[119] Besides, the alternative spliced isoform of BRAF [V600E,120] and constitutive bypass signaling such as YAP1,[121] amplification of cyclin D1,[122] loss of PTEN,[123,124] etc., were also implicated in this process.

Acquired mutations of MEK1 (Q56P, P124L and F129L)[125,126] and MEK2 (MEK2Q60P)[127] were reported to mediate the adaptive resistance to the combination of RAF

and MET inhibitors. These acquired mutations could increase ERK phosphorylation partly via MAP3K8 activation. Acquired splicing variants of BRAF^{V600E} (namely p61, p55, p48 and p41 based on their predicted molecular weight) exhibited enhanced dimerization that escaped the RAF inhibitor and activated the ERK signaling.[128–131] Acquired alterations in upstream components of the RAS–ERK pathway, such as elevated or mutant NRAS (Q61K),[132–134] RTKs (such as IGF1R and PDGFRβ[67,135]) and its ligands, BRAF amplification,[134,136,137] reduced NF1[138] and even elevated other RAF proteins (such as COT that activates MEK and ERK without BRAF),[139,140] and ERK-induced feedback (such as DUSPs and SPRYs) or potential MEK-induced non-ERK feedback inhibition, loss-of-function mutations in STAG2[141,142] and down-regulation of BOP1 (block of proliferation 1),[143] etc., are beneficial to the reactivation of the pathway, and those share similarity with the intrinsic resistance. Except for the acquired resistance of RAS–ERK pathway, acquired bypass activations should be considered as one of the vital factors of adaptive resistance to inhibitors of RAS–ERK signaling. For example, IL-6/STAT3 activation was reported to mediate the resistance to selumetinib (MEKi) in KRAS-mutant NSCLC cells.[144] Mutant mediator complex subunit 12 (MED12) contributed to the resistance to MEK and BRAF inhibitors via TGF-β signaling.[145] Also, the PI3K–AKT (gene mutation),[146,147] WNT5A/β-catenin,[148] and YAP1 signaling activations[121,149] are associated with resistance and promote tumor survival.

ERK inhibitors

ERK is found in more than 85% of cancers as well, which is majorly activated by the aberrant activation of upperstream kinase while no oncogenic ERK has been reported to our best knowledge. In contrast to the development of RAF and MEK inhibitors,[62] the current progress of ERK1/2 inhibitors is relatively limited. However, several ERK inhibitors are already in the clinical trials.

ONC201 (TIC10/NSC350625) induces TRAIL-mediated apoptosis in several tumors.[150] The considerable efficacy in cells and animals has led to the clinical development of ONC201. Animal studies showed no observed adverse event at 10-fold of expected therapeutic dose. After that, the first clinical trial in patients with refractory solid tumors showed that ONC201 was well tolerated, and the recommended phase II dose was 625 mg in an accelerated titration design.[151] Although no objective responses by RECIST were achieved, phase I/II clinical trials demonstrating the application of ONC201 in relapsed or refractory non-Hodgkin's lymphoma, metastatic triple negative breast cancer, relapsed or refractory acute leukemia or high-risk myelodysplastic syndrome, platinum refractory or resistant ovarian cancer, recurrent glioblastoma, or metastatic colorectal cancer is in full swing. In addition, ONC201 showed a good efficacy combined with lurbinectedin (a small molecule RNA polymerase II inhibitor), and received accelerated FDA approval in 2020 for metastatic small cell lung cancer that progressed after platinum-based therapy.[152] Future studies might explore the combination of ONC201 and lurbinectedin in more tumor types and the clinical translation in humans.

LY3214996 is a thieno [2,3-c] pyrrol-4-one compound, which selectively competes with the ATP of ERK, and it is synthesized by Cortez et al (United States patent US9469652. 2016.). For now, LY3214996 is applied as a single drug in healthy participant, acute myelocytic leukemia, unresectable or metastatic colorectal cancer, advanced/ metastatic cancer, and pancreatic cancer (NCT04033341, NCT04081259, NCT04616183, NCT02857270, and NCT04386057). The combinations of LY3214996 with SHP2 inhibitor (RMC-4630, NCT04916236) in KRAS-mutant cancers and with CDK inhibitor (abemaciclib, NCT04534283 and NCT04391595) in recurrent glioblastoma are being conducted by several groups. Preclinical studies showed that LY3214996 exhibited the single-agent activity, and resulted in synergistic (PI3K/mTOR inhibitor) and additive (CDK4/6 inhibitor) inhibition in patient-derived xenograft models of RAS-mutant lung cancer.[153] Moreover, LY3214996 enhanced the anti-tumor effects of RAF inhibitor (sorafenib) in hepatocellular carcinoma (HCC). These findings provide a theoretical basis for clinical trials of LY3214996 combined with PI3K/mTOR inhibitor, CDK4/6 inhibitor, or RAF inhibitor.[154]

Ulixertinib (BVD-523) is a highly selective, reversible ATP-competitive inhibitor of ERK1/2.[78] Clinical trials (NCT01781429, NCT02296242, and NCT02608229) indicated that ulixertinib has an acceptable safety profile with favorable pharmacokinetics. To date, ulixertinib has been explored in patients with lymphoma or melanoma. Notably, following that pharmacologically blocking both RAS—ERK and the autophagic process might be effective for pancreatic adenocarcinoma (PDAC), the efficacy of ulixertinib with hydroxychloroquine (an autophagy inhibitor) is being explored in patients with gastrointestinal adenocarcinoma and PDAC.[155,156] In addition, ulixertinib inhibits the tumor growth in NRAS- or BRAF-mutant xenograft models, and it potentiated the effects of gemcitabine, PI3K and HER inhibitors in several PDAC cell lines, and partly released ABCB1- and ABCG2-mediated chemotherapeutic drug resistance in cancer cells.[78,157]

MK-8353 is an oral and highly selective inhibitor of both active and inactive ERK1/2,[83] and can decrease the phosphorylation of RSK (an ERK1/2 substrate) that contributes to the ERK signaling inhibition as well.[84] Besides, MK-8353 is also a weak inhibitor of hERG. Preclinical studies showed the antitumor efficacy in several BRAF-mutant models. A phase I trial demonstrated antitumor activities in patients with metastatic BRAFV600-mutant melanoma.[158] However, there was no efficacy in advanced solid-tumor patients with NRAS- or KRAS-mutants, including NSCLC, PDAC and colorectal cancer.[158] Recently, a phase Ib trial was performed to evaluate the efficacy of MK-8353 in patients with advanced or recurrent solid tumors in combination with pembrolizumab (a humanized monoclonal anti-PD1 antibody)[88] and selumetinib (a MEK1/2 inhibitor) (NCT03745989).[159] Given that, further combination development of MK-8353 and selection of tumor types are currently under investigation.

GDC-0994 is an oral and highly selective inhibitor of ERK1/2 and inhibits the ERK phosphorylation, and could reduce the tumor growth *in vivo* and *in vitro*, including KRAS- and BRAF-mutant tumors.[160,161] A first-in-human phase I study demonstrated that GDC-0994 inhibited the MAPK signaling ranged from 19% to 51% with an acceptable safety profile, and induced 33% of overall responses of stable disease and 4% of partial responses in patients with BRAF-mutant colorectal cancer.[162] In a phase Ib study, GDC-0994 in combination with cobimetinib (a selective inhibitor of MEK1/2) was performed in patients with advanced solid tumors.[72] However, the trial was restricted because of the disability to manage the overlapping adverse events and cumulative toxicity.

Rineterkib, also known as LTT-462 or ERK-IN-1, is a RAF and ERK1/2 inhibitor that has demonstrated preclinical activity in multiple MAPK activated cancer cells and xenograft models. To date, rineterkib is applied as a single drug in adult patients with advanced cancers, including ovarian neoplasms, non-small-cell lung carcinoma, melanoma, etc., in phase I dose-finding study (NCT02711345),[73,74] with acceptable tolerance and limited clinical activity. Phase 1b and phase 2 clinical trials exploring the combination of LTT462 with RAF inhibitor (LXH254) in NSCLC and melanoma are ongoing (NCT02974725 and NCT04417621).[75] Except of that, a phase Ib trial is exploring the efficacy of combinations of LTT462 with different RAF inhibitors in colorectal cancer (NCT04294160). A multi-arm clinical trial is exploring the efficacy of LTT462 with JAK inhibitor (ruxolitinib).

Combination treatment of RAS—RAF—MEK—ERK pathway

Molecular target inhibitors provide a better way to customize cancer treatment for the minimum side effects on normal cells and improve efficacy. We have summarized the impact of these inhibitors alone on tumors. Next, we will describe the currently possible combination therapies including cytotoxic chemotherapy, radiotherapy, autophagy, and immunotherapy, which have been explored in preclinical experiments and clinical trials.

Combination with chemotherapy and radiotherapy

Radiotherapy (RT) is applied in approximately 50% of all cancer patients with about 40% of the curative efficacy. The primary mechanism of RT-induced anticancer toxicity is DNA double-strand breaks (DSBs), while some tumors could oppose to the efficiency through DNA damage repair. ERK and CRAF mediate RT-induced repair of DNA damage and promote tumor resistance to RT.[76,77] MEK inhibitor (GSK1120212) significantly suppressed the DSB repair and sensitized the KRAS-mutant pancreatic tumor cell lines to RT. RAF inhibitors have similar effects that vemurafenib sensitized BRAF[V600E] thyroid cancer cells to RT by reducing the DSB repair. In contrast, PLX4720 sensitized high-grade BRAF[V600E]-mutant glioma cells to RT.[79,80] Vemurafenib also significantly enhanced melanoma cell radio-sensitivity and synergistic tumor inhibition in melanoma xenograft models.[81]

Clinically, an RAF inhibitor (sorafenib) and radiotherapy were administrated to hepatocellular carcinoma patients.[82] However, severe skin toxicity was observed after receiving BRAF inhibitors (vemurafenib and dabrafenib) and RT[85] except for the report of Rompoti.[86] And no obvious intra-cranial toxicity was found in combined treatment compared with that of RT alone. These phenomena might be related

to the distinct metastatic locations that accepted RT. A large multi-center analysis was carried out to generate reliable data, and an acceptable toxicity was observed after radiotherapy with concomitant BRAF inhibitor therapy.[87] Recently, the melanoma patients receiving interruption of vemurafenib treatment during radiation showed a prolonged survival and lower toxicity than those receiving concomitant treatment.[89] The RAF/MEK inhibitors, which can penetrate the blood—brain barrier, are considered to treat melanoma brain metastasis (NCT02039947)[90] combined with RT, and considerable efficacy and manageable safety were achieved. Selective BRAF and MEK inhibitors could also re-sensitize the advanced thyroid cancer cells to RT and induce a better response in patients, but the adverse events are largely remained unknown.[91] These studies imply more focus on the combination in different tumors.

Chemotherapy resistance is another issue for the refractory tumors.[92] The RAS—RAF—MEK—ERK pathway is widely activated in tumors, and high frequency of KRAS and BRAF mutations are observed in pancreatic cancer, melanoma and lung cancer.[93,107,108] A recent study has declared the association between RAS—RAF—MEK—ERK pathway and cytotoxic chemotherapy resistance. The combination of RAF inhibitors (sorafenib) and traditional chemotherapy regimens (gemcitabine) did not show significant advantages in high-grade pancreatic cancer.[93] Also, the combination of MEK inhibitor (binimetinib) and gemcitabine did not improve the disease-free survival time in cholangiocarcinoma patients. These studies suggested that combining RAS—RAF—MEK—ERK inhibitors and gemcitabine might be unable to improve the treatment outcome. However, this pathway combined with platinum-based chemotherapy has a good therapeutic effect. For example, the combination of MEK inhibitor (MEK162), bevacizumab and paclitaxel improved the treatment outcome without additional adverse events in ovarian cancer.[94] Inhibition of ERK augmented the cisplatin sensitivity in squamous cell carcinoma cells and demonstrated the efficacy and acceptable tolerability in vivo.[163] A phase II clinical trial evaluated the safety of a MEK inhibitor (selumetinib) combined with first-line platinum inhibitors (carboplatin and cisplatin), and suggested that selumetinib combined with platinum drugs has more potential than platinum alone in NSCLC.[164]

Combination with autophagy inhibitors

Recently, autophagy has been reported to act as an accomplice that contributed to the resistance to RAS—RAF—MEK—ERK pathway inhibitors especially in BRAF^{V600E}-mutant tumors. Sorafenib, a multi-kinase inhibitor of the RAS—RAF—MEK—ERK pathway, has been applied to treat advanced HCC. But the resistance has obstructed its application, partly because of an accumulation of autophagosomes.[165] Concomitant inhibition of autophagy inhibited the growth of tumors in mice, which uncovers the potential of this combination in HCC therapy. Consistent with sorafenib, resistance occurred after a period of treatment with UI-152 (an inhibitor of BRAF) in melanoma[166,167]; autophagy inhibition partially arrested the cell growth and promoted tumor regression after treatment

with UI-152.[168] The newly developed autophagy inhibitor also sensitized the BRAF^{V600E}-mutant melanoma cells to vemurafenib.[167,169] In BRAF^{V600E}-mutant glioma cells, autophagy inhibition augmented the growth inhibition accompanied by BRAF inhibitor (UAI-201).[170] Similar synergistic effects are also demonstrated in other BRAF-mutant brain tumors, thyroid cancer, pancreatic cancer and colorectal cancer.[171—176] Combining MEK inhibitors and autophagy inhibitors allowed BRAF-resistant cells to die again.[177] Although the combination of inhibitors of autophagy and RAS—RAF—MEK—ERK pathway has shown good potential in cells and mouse models, the relevant clinical trials are still in an exploring phase. The above studies provide a good theoretical basis for the clinical trials that combining RAS—RAF—MEK—ERK inhibitors with autophagy inhibitors.

Combination with immune inhibitors

Immunotherapy has made remarkable progress in achieving an extension of overall survival and long-term durable remission in tumor patients, which mainly depends on memory CD8^+ T cells. Recently, studies have shown that MEK inhibition could reprogram the CD8^+ T cells into memory stem cells with potent antitumor ability,[178] and RAF inhibition could induce PD-L1 expression of cancer cells.[179—181] According to the biopsies and gene-based transcriptomic analysis, BRAF inhibitors were found to induce the exhausted CD8^+ T cells in resistant melanomas.[182,183] In triple-negative breast cancer cells, the MEK inhibitor significantly increased the expression of PD-L1; combining PD-L1 antibodies and MEK inhibitor enhanced the antitumor immune response in a mouse model of breast cancer and colon cancer.[180,181] In pancreatic cancer, the MEK inhibitor acted on the myeloid cells of the immunosuppressive environment and enhanced the efficiency of PD-L1 antibodies.[184] Genomic landscapes of gastroesophageal adenocarcinoma provided a novel insight that combined these pathway inhibitors with a specific immune checkpoint inhibitor.[185] A recent study also revealed that a RAS mimetic, namely rigosertib, augmented response to checkpoint blockade by inducing the expression of CD40.[186] All the evidence has driven the progress of the clinical trials that combining RAS—RAF—MEK—ERK inhibitors with immunotherapies.

Further, short-term treatment (less than 15 days) of combination of BRAF/MEK inhibitors and anti-PD-1/PD-L1 antibodies increased the immune cell infiltration and arrested the tumor growth in melanoma patients.[187] However, a phase I study was terminated because of the dose-limiting hepatotoxicity in patients who received vemurafenib and ipilimumab.[188] Another phase I study explored the clinical effect and safety of combination of anti-PD-L1 antibody and BRAF inhibitor (dabrafenib)/MEK inhibitor (trametinib) in BRAF-mutant and BRAF wild-type melanoma (NCT02027961).[189] The clinical efficacy was considerable, and further investigations such as longer follow-up and more patients should be conducted for the safety evaluation of the concurrent treatments. Besides, a phase II study explored the efficacy of combination of anti-PD-L1 antibody (pembrolizumab) and BRAF/MEK inhibitors (dabrafenib and trametinib) in advanced melanoma patients (NCT02625337),[190] and suggested a

potential for optimization of the administration order in the treatment of melanoma patients.

In recent years, immune checkpoint inhibitors have uncovered a new era for lung cancer treatment,[191] but the overall remission rate are still lower than 31%.[192,193] Preclinical data showed that T cell activation and cytotoxic T cell-associated antigen 4 (CTLA-4) expression increased after the combination treatment of selumetinib and trametinib. In addition, pulsed MEKi therapy combined with CTLA-4 blockade prolonged the survival of tumor-bearing mice with KRAS mutation.[194,195] Therefore, an initial phase Ib study was performed to explore the safety and efficacy of cobimetinib combined with atezolizumab. The median OS time for NSCLC was 13.2 months, and the ORR reached to 18%. The most common adverse events included diarrhea (67%), skin rash (48%) and fatigue (40%).[196] Another ongoing phase I/II trial aims to evaluate the continuous or intermittent administration of selumetinib for immunotherapy (durvalumab/tremelimumab) in patients with NSCLC.[197] Therefore, the clinical data on MEK inhibitors combined with immune checkpoint inhibitors are still not enough to determine the best remedy for NSCLC.

Conclusions and future perspectives

Inhibitors targeting RAS—RAF—MEK—ERK exhibit good tumor-suppressive effects and biosafety. Various types of inhibitors have been approved by FDA thus far, but long-term administration leads to the emergence of drug resistance. The major mechanisms of RAFi and MEKi resistance are the re-activation of the RAS—ERK pathway and activation of the bypass signal pathway, therefore targeting the downstream molecule and bypass activation might provide an alternative strategy. At present, the exploration of inhibitors targeting ERK has been in full swing, that several inhibitors have been under evaluation in clinical trials, even so, no drugs have been approved. In addition, the combinations of ERKi with RNA polymerase II, CDK, PI3K, hydroxychloroquine, or up-stream molecules inhibitors have been explored in several clinical trials, however, severe adverse events are observed after the treatment with MEKi, which suggested that combinations with bypass signaling pathway blocking might be a better choice than the addition of the same pathway inhibitors. Except for the combinations with other targeted therapies, inhibitors of the RAS—ERK pathway alleviate resistance to traditional radiotherapy and chemotherapy and can enhance the therapeutic effects of antibodies (targeted PD-1, PD-L1, and CTLA-4). For now, the efficacy of RAFi/MEKi with traditional therapies has been well investigated and high levels of activated ERK are always observed in cisplatin and RT resistant tumor cells,[198–202] while the combinations with ERK inhibitors are still rarely known.

Taken together, further studies should highlight the extra drug-resistance mechanisms, especially the additional activation of the bypass signaling, and focus on the therapeutic schedule for each specific tumor, including optimal combinations of inhibitors and/or clinically used drugs, suitable dosage, and even different groups of patients, therefore obtaining a considerable efficacy with minimum adverse events.

Author contributions

Conflict of interests

The authors declare no competing interests.

Funding

Acknowledgements

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