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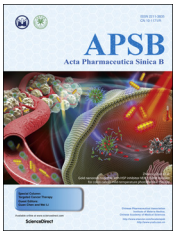

REVIEW

Targeting ERK, an Achilles' Heel of the MAPK pathway, in cancer therapy

Feifei Liu, Xiaotong Yang, Meiyu Geng, Min Huang*

Division of Antitumor Pharmacology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

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Abstract The mitogen-activated protein kinases (MAPK) pathway, often known as the RAS-RAF-MEK-ERK signal cascade, functions to transmit upstream signals to its downstream effectors to regulate physiological process such as cell proliferation, differentiation, survival and death. As the most frequently mutated signaling pathway in human cancer, targeting the MAPK pathway has long been considered a promising strategy for cancer therapy. Substantial efforts in the past decades have led to the clinical success of BRAF and MEK inhibitors. However, the clinical benefits of these inhibitors are compromised by the frequently occurring acquired resistance due to cancer heterogeneity and genomic instability. This review briefly introduces the key protein kinases involved in this pathway as well as their activation mechanisms. We also generalize the correlations between mutations of MAPK members and human cancers, followed by a summarization of progress made on the development of small molecule MAPK kinases inhibitors. In particular, this review highlights the potential advantages of ERK inhibitors in overcoming resistance to upstream targets and proposes that targeting ERK kinase may hold a promising prospect for cancer therapy.

*Corresponding author.
E-mail address: mhuang@simm.ac.cn (Min Huang).

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1. Overview of the MAPK pathway

The mitogen-activated protein kinases (MAPK) pathway, often known as a cascade of protein kinases composed of RAS, RAF, mitogen-activated protein/extracellular signal-regulated kinase (MEK) and the extracellular signal-regulated kinase (ERK), is a highly conserved signal transduction pathway in all eukaryotic cells. The MAPK pathway is one of the best-characterized signaling cascades that regulates a variety of normal cellular functions, such as cell proliferation, differentiation, survival and apoptosis, by transmitting signals from upstream extracellular growth factors to diverse downstream effectors located in the nucleus[1]. The activation of the MAPK pathway initiates from a conformational change of RAS. Stimulated by upstream receptors, guanosine diphosphate (GDP)-bound RAS (inactive) switches to guanosine triphosphate (GTP)-bound RAS (active), which causes the recruitment and activation of RAF. Activated RAF phosphorylates and actives MEK, whose activation directly leads to the phosphorylation of ERK. Activated ERK phosphorylates multiple substrates ranging from kinases to transcription factors, and is positioned as a key kinase that controls a large number of cellular processes due to its rather broad nature of substrate recognition.

1.1. *Components of the MAPK pathway*

The principal upstream factor of the MAPK pathway is RAS protein, a member of the small GTPase (guanosinetriphosphatases) superfamily composed of more than 150 members. Members of the RAS superfamily are divided into families and subfamilies based on their structure, sequence and function. The five main families are RAS, RHO, RAN, RAB and ARF GTPases (Fig. 1). The RAS family itself is further divided into 6 subfamilies (RAS, RAL, RAP, RAD RHEB, and RIT) and each subfamily shares the common core G domain, which provides essential GTPase and nucleotide exchange activity. RAS is the most frequently studied protein in the RAS subfamily. In humans, four RAS proteins have been identified, including HRAS, NRAS, KRAS4A and KRAS4B. The KRAS4A and KRAS4B are two isoforms of KRAS, produced by alternative splicing of the same gene[2]. RAS is a GTP-binding protein and serves as a molecular switch in a cycle between inactive RAS–GDP and active RAS–GTP[3]. The GDP/GTP cycle is regulated by RAS guanine nucleotide exchange factors (GEFs) such as Son of Sevenless (SOS1) protein that catalyze the formation of RAS–GTP[4]. Meanwhile, GTPase hydrolyzes RAS–GTP to RAS–GDP and consequently terminates the RAS signaling, accelerated by the interaction of GTPase with GTPase-activating proteins (GAPs) including p120GAP and neurofibromin[4]. In response to extracellular stimuli, inactive RAS–GDP converts to active RAS–GTP to promote the activation of several downstream effectors. Activation of RAS stimulates various signaling pathways, which includes the MAPK pathway, the PI3 kinase (PI3K) pathway and the Ral-GEFs pathway; among them the MAPK pathway is the best characterized[5].

RAF is the first protein kinase of the MAPK pathway and has three isoforms which are the paralogues ARAF, BRAF and CRAF, sharing a high similarity of domain organization[6]. Active RAS–GTP binds to the N terminus RAF and leads to activation of RAF[7]. RAF activation involves an array of complex processes that include recruitment of RAF from plasma, the formation of a RAS–RAF complex in the membrane, dimerization of RAF proteins, phosphorylation or dephosphorylation of different domains of RAF, disassociation from RAF kinase inhibitory protein (RKIP) and association with scaffolding complex such as kinase suppressor of RAS (KSR)[4,8]. The precise sequence of these events has not been well elucidated, but the RAF dimerization is widely considered a pivotal step[9]. After being activated, RAF protein phosphorylates and activates MEK, followed by the positive phosphorylation of ERK1/2. All three isoforms of RAF are able to active MEK1/2 through phosphorylation, while B-RAF shows the most potent activity[9].

MEK is a central component in the MAPK signaling cascade. MEK1 and MEK2 are tyrosine (Tyr) and serine (Ser)/ threonine (Thr) dual-specificity kinases and share approximately 80% similarity. Within the MAPK pathway, MEK1/2 are phosphorylated and activated by RAF[10]. However, RAF is considered only a subset of MEK1/2 activators, as MEK1/2 are also activated by multiple MAP kinase kinase kinases (MAP3Ks) including MEKK1 (mitogen-activated protein kinase kinase kinase 1), MEKK2/MEKK3 component, MAP3K8 and the mixed-lineage kinases (MLK1–4; also known as MAP3K9, MAP3K10, MAP3K11 and KIAA1804)[11–14]. The signals of these upstream activators converge at the level of MEK1/2. On the contrary, MEK1 and MEK2 are the exclusively specific activators of ERK1/2. Since ERK1/2 have been discovered to regulate a large number of substrates, MEK1/2 serve a unique role as crucial "gatekeepers" in MAPK cascade[10].

ERK1 (p44) and ERK2 (p42) are the proteins encoded by two splice variants of the same gene, and members of the MAPK superfamily also include ERK3/4, ERK5, ERK7/8, Jun N-terminal kinase (JNK)1/2/3 and p38, α, β and γ (ERK6) and δ[15,16], all of which have been shown to play roles in cancer. ERK1/2 can be positively activated by MEK1/2 through phosphorylation of Thr and Tyr residues, namely Thr202 and Tyr204 of ERK1 and Thr173 and Tyr185 of ERK2, respectively. Previous studies have suggested that ERK1 and ERK2 may have distinct functions for proliferation, and RAS-induced transformation requires ERK2 rather than ERK1. ERK1 has been discovered to antagonistically compete with ERK2 for MEK, which results in a weakening of ERK2 signaling. It still remains unclear whether ERK1 and ERK2 have different substrates[17]. Inactive ERK1/2 are associated with microtubules in the cytoplasm. Upon being phosphorylated by MEK1/2, they translocate into the nucleus and activate a substantial variety of transcriptional factors[18]. ERK1 and ERK2 have hundreds of substrates, many of which participate in key physiological processes that control cell proliferation, differentiation, survival and death[19]. The phosphorylation and activation of transcriptional factors, including CREB, ELK-1, ETS, NF-κB, c-Myc, and the stimulation of the 90-kDa ribosomal S6 kinase (p90RSK) (Fig. 2). The association with scaffold PEA-15A to enhance ERK activity is one of a few examples showing that ERK1/2 regulates their substrates to carry out normal cellular functions[19–22]. Additionally, the activation of ERK substrates can lead to feedback loops, which in turn regulate the ERK signaling pathway either positively or negatively depending on the substrate[23].

1.2. *Activation of the MAPK pathway*

The activation of the canonical MAPK pathway is triggered by a stimulating process in which the growth factors (such as EGF) bind to the cell-surface receptors mainly tyrosine kinase receptors (RTK, such as EGFR), resulting in the dimerization and trans-phosphorylation of RTK[24]. This binding of growth factors to cell-

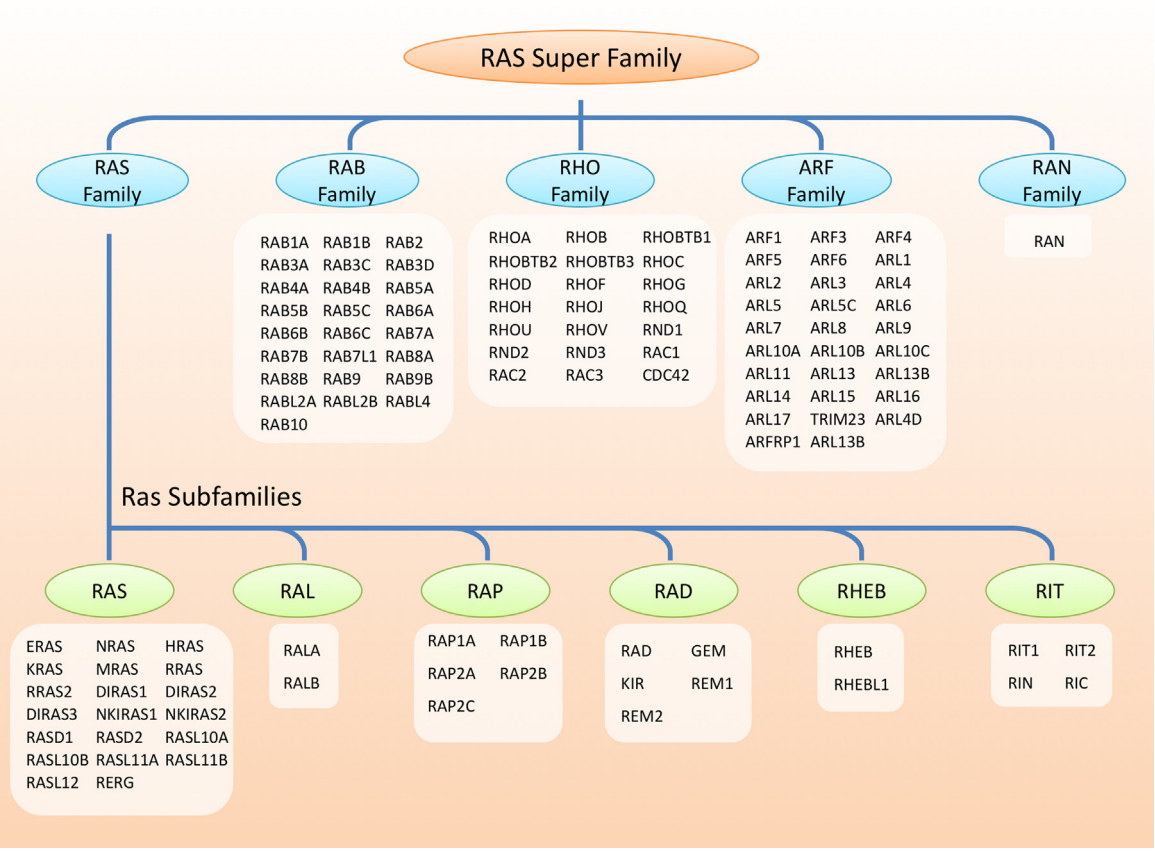

Figure 1 The major RAS family numbers. The RAS GTPase superfamily is composed of five main families, RAS, RHO, RAN, RAB and ARF. The Ras family itself is further divided into 6 subfamilies, RAS, RAL, RAP, RAD, RHEB and RIT.

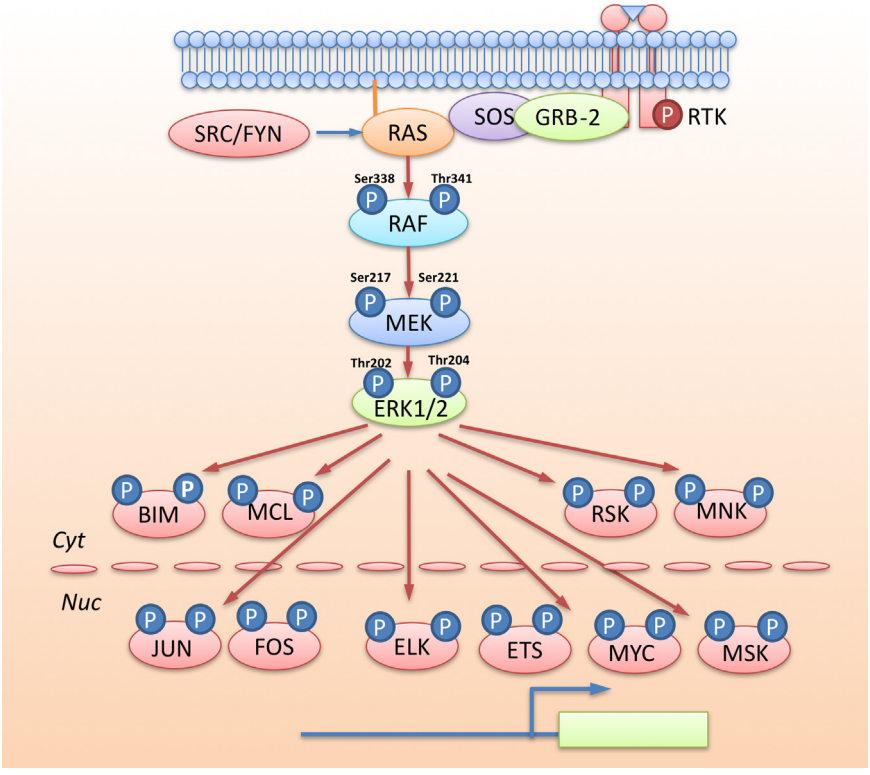

Figure 2 The major downstream targets of ERK1/2 in the MAPK pathway. ERK regulates both cytosolic targets and nuclear transcription factors, thus promoting proliferation, survival and other malignant phenotypes.

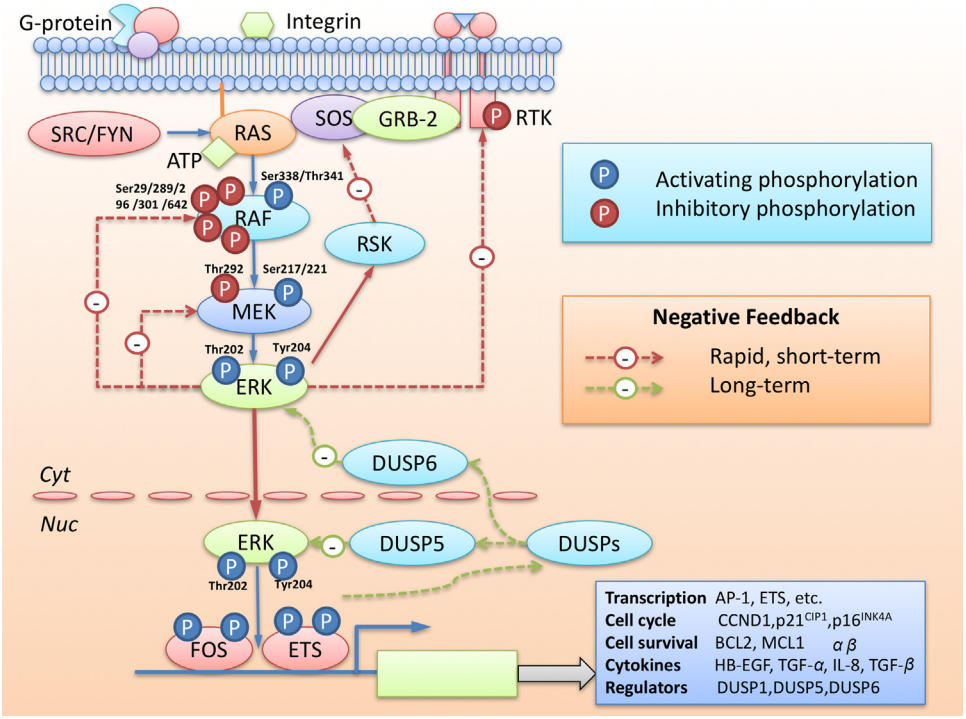

Figure 3 Activation and feedback regulation of the MAPK pathway. The classical MAPK pathway is activated in human tumors by upstream receptor tyrosine kinases (RTK) or by mutations in RAS, BRAF, and MEK1. RTKs activate RAS by recruiting adaptor proteins (*e.g.*, GRB-2) and exchange factors (*e.g.*, SOS). RAS activation promotes the formation of RAF dimers, which activate MEK-ERK cascade through phosphorylation. ERK pathway activity is regulated by negative feedback at multiple levels, including the transcriptional activation of DUSP proteins that negatively regulate the pathway. ERK also phosphorylates and thus regulates CRAF and MEK activity directly. ERK, or its immediate substrate RSK, also phosphorylates SOS at several residues, inhibiting its activity and thus negatively regulating RAS activity.

surface receptors causes the formation and activation of receptor complexes which contain adaptors including SHC (SH2-containing protein), GRB2 (growth-factor-receptor bound protein 2) and GAB (GRB2-associated binding) proteins. Among these proteins, those containing SH2 domains are recruited to specific phosphotyrosine residues. One of these SH2-containing proteins, GRB2, constitutively binds to the RAS activator SOS[5]. Adaptor proteins associate with the RTK-phosphorylated intracellular domains, which can recruit GEFs to cell membrane, increasing the level of RAS–GTP bound protein[4]. RAS–GTP is demonstrated to directly bind to RAF protein, recruiting RAF from the cytoplasm to membranes, which enables the RAF to be an active kinase[8,25]. Activated RAF subsequently carries out a chain of phosphorylation reactions to its downstream substrates, namely, MEK and ERK, initiating the activation of the canonical MAPK pathway[7].

The ERK cascade is under extensively homeostatic control by feedback loops (Fig. 3), the effects of which can be broadly divided into two different types: rapid short-term effects and a delayed long-term effect[26]. The rapid feedback mechanisms refer to activated ERK1/2 in turn stimulating inhibitory phosphorylation of their upstream factors and kinases such as MEK, RAF, SOS and RTKs, which prevents signal propagation of this pathway and maintains stable cellular functions[18,27]. Specifically, ERK1/2 phosphorylate BRAF and CRAF to inhibit the phosphorylation of MEK[28]. The delayed feedback mechanisms can be briefly described as the *de novo* expression of Sprouty (SPRY) proteins and dual-specificity phosphatases (DUSPs) that regulate the MAPK pathway by dephosphorylating ERK1/2. Namely, SPRY proteins interfere with a RAF catalytic domain to inhibit RTKs and SOS, while DUSPs dephosphorylate the p-T-E-pY motif to inactive ERK[29]. In short, the feedback loops control the ERK signaling pathway in multiple ways, and play an essential role in maintaining cellular homeostasis in physiological conditions.

2. Aberrations of the MAPK pathway in cancer

It has been widely appreciated that aberrant activation of this pathway is closely linked to various kinds of cancers. Dysregulated MAPK signaling leads to the occurrence and progression of cancers *via* multiple mechanisms, particularly gentic alterations[1,26]. *RAS* has been identified as an oncogene and is mutationally activated in approximately one-third of all cancers, with pancreas (90%), colon (50%), thyroid (50%), lung (30%) and melanoma (25%) with high-ranking prevalence[30]. *RAS* mutants encode mutated proteins that harbor single amino-acid substitutions primarily at glycine 12 (G12) and glutamine 16 (Q16) in human cancers (Table 1). These mutated proteins are GAP-insensitive and constitutively GTP-bound, leading to stimulus-independent and persistent activation of the downstream effectors. Among the *RAS* family, *KRAS* is the most frequently mutated isoform and occurs in more than 20% of all human cancers, followed by *NRAS* (8%) and *HRAS* (3.3%), and no other *RAS* mutation has been found[30]. The mutation types of RAS

Table 1 MAPK mutations in different cancers.

Cancer type	Mutation type and rate (%)	Major mutation site
Prostate cancer	KRAS (90%)	G12D, G13D, G12V, G12S, G12C
NSCLC	NRAS (35%)	Q61K, Q61R, C186F, Q61L, Q61K,
CRC	KRAS (45%)	G12D, G12V, G13D, G12C, A146T, F566L
	BRAF (12%)	V600E
Pancreatic cancer	KRAS (90%)	G12D, G12V, G12R, G12C,
Melanoma	NRAS (15%)	Q61R, Q61L, Q61K, Q61H
	BRAF (66%)	V600E
Bladder cancer	KRAS (50%)	G12V, G12D, G12C,
AML	NRAS (30%)	G12D, G13D, G12V, Q61H, A59E, A164T
Ovarian Cancer	BRAF (30%)	V600E, A747V, G464E, V226M
Papillary thyroid cancer	RAS (60%)	KRAS:G12R, NRAS:Q61R
	BRAF (35%–70%)	V600E

NSCLC, non-small cell lung cancer; CRC, colorectal cancer; AML, acute myeloid leukemia.

proteins may be associated with tumor types; the *NRAS* mutations have been identified in approximately 20% of melanomas, for example [31].

BRAF mutations have been widely identified in tumors, with a significant percentage (7%) of all human cancers. This mutation is highly prevalent in hairy cell leukemia (100%), melanoma (50%–60%), papillary thyroid cancer (40%–60%), colorectal cancers (CRC, 5%–10%), pilocytic astrocytoma (10%–15%) and non-small cell lung cancer (NSCLC, 3%–5%)[32]. The most common mutation of BRAF refers to BRAF-V600E, which is a point mutation at valine 600 to yield glutamic acid. The BRAF-V600E mutation is notably prevalent in melanomas (63%) and papillary thyroid carcinomas (more than 50%)[33]. Oncogenic *BRAF* mutations lead to overactivity of its downstream effectors MEK and ERK[32].

In terms of downstream kinases in the MAPK pathway, MEK mutations have been mainly identified in melanoma[10], and also in ovarian cancer cell lines and gliomas[34,35]. Generally, all of the upstream mutations can lead to ERK protein hyperactivation, which is responsible for a series of ERK-signaling-regulated substrate activation and consequently related to a wide range of tumors[36]. For instance, overexpression of ERK can induce modulation of anti-apoptotic molecules such as BCL-2, a protein that is linked to drug resistance in some types of breast cancer[37].

3. Inhibitors targeting the MAPK pathway

Targeting the MAPK pathway has attracted significant interest in cancer therapy. Efforts directly targeting RAS protein are believed to be very challenging in spite of the promise shown by a few RAS inhibitors in the early development stage. Clinical benefits achieved by BRAF and MEK inhibitors have shown that targeting these downstream RAS effectors is a very promising approach for therapies of cancers harboring oncogenic mutations in this pathway[1]. However, patients treated with RAF or MEK inhibitors frequently develop drug resistance. The resistance involves very complicated mechanisms, including gene mutations occurring in the targeted proteins, MAPK signaling interaction with PI3K pathway, loss of functions in MAPK signaling feedback control and abnormal alterations of tumor suppressor genes[38]. It has been believed that single drug resistance can trigger multi-drug resistance[39]. Given this situation, researchers continue to pursue approaches that can reverse drug resistance, and develop combinatorial strategies to obtain therapeutic efficacy[40,41].

3.1. *Efforts in targeting RAS protein*

RAS protein is a central regulator of growth factor-induced cell proliferation and survival in cells, and is the upstream factor of the ERK signaling pathway, PI3K pathway and Ral–GEFs pathway[41]. The aberrant activation of RAS is closely linked with various kinds of cancers. It is a significant challenge to develop RAS inhibitors, and three decades of this effort has not generated clinically effective molecules so far[31]. This difficulty is firstly attributed to the tertiary structure of RAS protein, which is very smooth and floppy, thus hardly providing a binding pocket for small molecule inhibitors[42]. Moreover, oncogenic mutant RAS proteins are insensitive to GTPase-activating protein-catalyzed GTP hydrolysis, resulting in constitutively active GTP-bound protein. The affinity of RAS protein for GTP is extraordinarily high, and it is almost impossible to develop a competitive binding strategy as a result[43]. These characteristics of RAS make directly targeting it very hard to achieve, so that recent targeting strategies are mainly on proteins that regulate RAS–GTP interaction or RAS mutants. Other efforts also explore the therapeutic opportunity of targeting SOS[44], which plays a role in regulating the rate of GDP/GTP exchange. Small molecules have been discovered to be able to bind a unique pocket on RAS–SOS–RAS complex, which facilitates SOS-catalyzed nucleotide exchange and interferes with MAPK signaling[45].

Recently, a strategy that targeted KRAS mutation G12C gained increasing interest. The G12C mutation refers to a glycine replaced with a cysteine and it occurs frequently in lung cancers, roughly 50% of RAS-driven lung adenocarcinomas. Lim et al.[46] reported that small molecule compounds, SML-8-73-1 and SML-10-70-1, can selectively inhibit KRAS G12C mutant. Biochemical analysis has shown that the inhibition of KRAS G12C with SML-8-73-1 restrains KRAS protein in an inactive state. Crystallographic studies demonstrate that the inhibition of KRAS G12C disrupts the effector binding regions switch-I and switch-II, which subverts the native nucleotide binding preference from GDP to GTP. This study validates KRAS-G12C as a targetable mutant.

Recruitment of RAS to the membrane is a crucial step of RAS activation. This process requires a post-translational modification

such that farnesyl transferase adds a lipid tail on RAS, enabling it to attach to the cell membrane. Targeting this transferase to prevent RAS membrane recruitment was originally considered a promising approach. This approach eventually failed as no farnesyl transferase inhibitors present positive clinical effects[47]. An explanation proposes that different isoforms of RAS protein such as NRAS and KRAS, can be geranylgeranylated if the farnesyltransferase is inhibited, and these prenylated RAS proteins retain the ability to localize to the cell membrane and are still functional[30]. Recently, it has been revealed that correct localization and signaling of farnesylated KRAS is regulated by prenyl-binding protein phosphodiesterase δ (PDEδ), which sustains the spatial organization of KRAS by facilitating its diffusion in the cytoplasm. The strategy of targeting tPDEδ has shown activity in suppressing RAS activity and anticancer activity has been observed in animal models[48].

In short, RAS protein was believed to be "undruggable" due to the aforementioned reasons. Recent novel approaches that target RAS–GTP interactions or the KRAS mutation are considered promising strategies[49]. However, the effect of suppressing MAPK signaling by these approaches is still limited to laboratory models and is far from showing clinical effectiveness.

3.2. *BRAF inhibitors and the BRAF paradox*

As the downstream kinase of RAS in MAPK cascade, the RAF family proteins play an important role in this signal transduction[9]. Among the three RAF isoforms, *CRAF* was first identified as an oncogene and considered a potential target, as CRAF is documented as an important RAS effector[50]. Efforts in targeting CRAF have yielded numerous pre-clinical compounds. In particular Sorafenib (Nexavar, Bayer/Onyx), an orally-available compound that was originally discovered as a CRAF (also BRAF) inhibitor and lately identified to be a multikinase inhibitor, was approved for renal and hepatocellular carcinoma[51,52], for its anti-angiogenesis effect.

Later efforts have highlighted the promise of targeting BRAF for the treatment of BRAF-mutant melanoma, which validates BRAF as a therapeutic target. Selective BRAF inhibitors, such as vemurafenib and dabrafenib, have achieved clinical benefits[53,54]. Vemurafenib (Zelbraf, Roche/PLexxikon) was the first BRAF-V600E-selective inhibitor that entered clinical trials. It was approved for metastatic and unresectable *BRAF*-mutated melanomas[55]. Dabrafenib (Tafinlar; GlaxoSmithKline) was approved for *BRA*F V600K-mutated metastatic melanoma in 2013[53]. Vemurafenib and dabrafenib gained desirable clinical efficacy in the treatment of patients suffering *BRAF-V600E* and *BRAF-V600K* melanomas, which yielded a significant improvement in disease-free progression and overall survival of these patients.

Though BRAF inhibitors have achieved clinical benefits in the treatment of melanomas, the extent and duration of treatment with BRAF inhibitors is variable and the extremely high frequency of emergence of drug resistance eventually leads to failure of the treatment using BRAF inhibitors[56]. For example, recent studies have shown that all ATP-competitive RAF inhibitors, including vermurafenib, dabrabenib and sorafenib, could lead to paradoxical activation of the MAPK pathway in *BRAF* wild-type cells. The paradoxical activation of MAPK pathway is an intriguing phenomenon that primarily results from conformational changes such as RAF dimerization and transactivation enhanced by RAF inhibitors[57,58]. This paradox causes various degrees of adverse effects, which are mainly benign skin tumors including squamous cell carcinomas and keratoacanthomas[59]. The paradox-induced skin tumors have an uncharacteristically high incidence of *RAS* mutations, raising the concern that the same mechanism might accelerate progression of other RAS-driven cancers. Also, studies demonstrated that paradoxical activation of ERK signaling can promote tumor growth in both *RAS*-mutated tumors and BRAF tumors[60].

Furthermore, efficacy of selective BRAF inhibitors is limited to *BRAF*-mutated metastatic melanoma, despite the fact that the *BRAF* mutation has been identified extensively in carcinomas such as CRCs, thyroid cancers, glioblastoma and NSCLC[60]. Facing the challenges of drug resistance and paradoxical activation encountered in the treatment of BRAF inhibitors, researchers attempt to seek solutions with combination usage of inhibitors of BRAF and other pathways. There are reports suggesting that the insensitivity to RAF inhibitors could be driven by the EGFR-mediated MAPK signaling reactivation in *BRAF*-mutant CRCs[61]. Combination of EGFR and BRAF inhibitors in *BRAF* mutant CRC cells have been shown to be able to block the reactivation of MAPK signaling and improve the therapeutic efficacy *in vivo*[62].

3.3. *MEK inhibitors*

The MEK1/2 kinases in the MAPK pathway were not considered potential targets in the past, as MEK1/2 are rarely mutated in human cancers[63]. Lately, with the emergence of the paradoxical phenomenon of the first generation of RAF inhibitors, targeting MEK1/2 has attracted growing interest among pharmacological researchers[10,64]. The first MEK1/2 inhibitor PD098059 was reported in 1995, which was shown to inhibit the dephosphorylated form of MEK1 and MEK1 mutant (S217E, S221E). This compound is an allosteric inhibitor and its discovery indicated that MEK1/2 are valuable cancer drug targets[65]. Trametinib (MEKinist™, GlaxoSmithKline/Japan Tobacco) is the first MEK inhibitor to reach the market, approved as a single agent for BRAF V600E metastatic melanoma in 2013[66]. Furthermore, targeting *KRAS* mutant cancers with dual inhibitors is under extensive study and recently shown to be effective[67]. The combination of MEK inhibitors and PI3K-AKT pathway inhibitors was found to increase progression-free-survival (PFS)[10,67]. Also, the combination of MEK inhibitors and first-generation BRAF inhibitors are better tolerated than the respective mono-therapies, because the paradoxical activation of MAPK signaling in BRAF wild-type tissues antagonizes the inhibitory functions of MEK inhibitors, and in turn limits this paradoxical activation[67]. FDA also approved the combination of dabrafenib and trametinib for *BRAF-V600E/K*-mutant metastatic melanoma in 2014. The combination of the RAF inhibitor with the MEK inhibitor cobimetinib[40] (GDC-0973, Genentech) led to simultaneous suppression of both the melanoma and RAF-inhibitor-induced leukemia.

However, as with other small molecule inhibitors, patients treated with MEK inhibitors also develop drug resistance within several months[10]. The primary sensitivity of MEK inhibitors correlates with the decreased expressions of cyclin D1 (*CCND1*), p27 (*KIP1*) and cell cycle arrest[63]. In the presence of MEK inhibitors, feedback reactivation of MAPK signaling seems to be

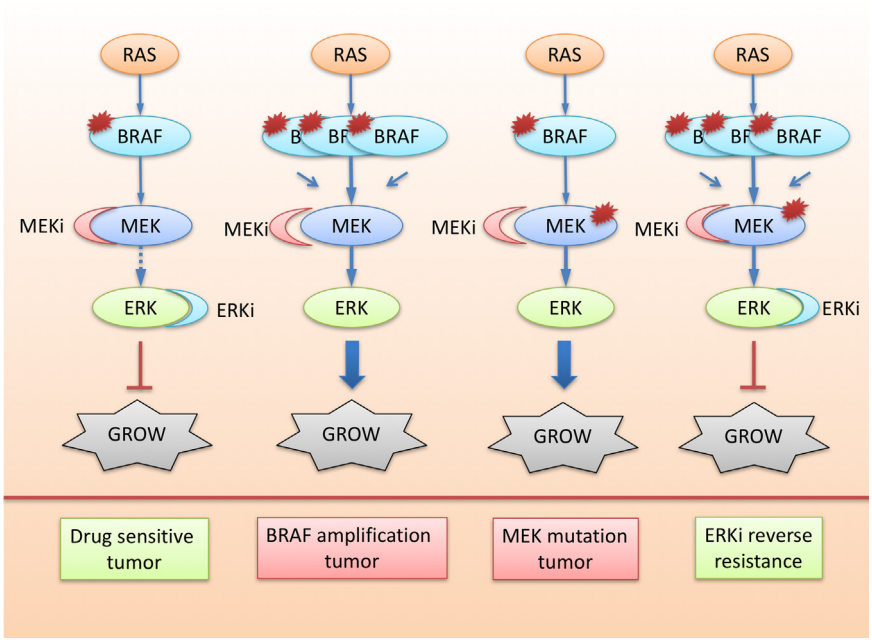

Figure 4 Therapeutic potential in cancers that are resistant to MEK and BRAF inhibitors. Resistance to BRAF inhibitors can occur through various mechanisms, including activating *BRAF* mutations and *BRAF* amplification, which can be overcome by both MEK inhibitors (MEKi) and ERK inhibitors (ERKi). ERK inhibitors have the advantage to further overcome resistance to MEK inhibitors that occurs upon MEK mutation.

consistently stronger in *RAS* mutant tumors than *BRAF-V600E* tumors, with reasons that are not entirely understood. ERK feedback regulation is also considered to be related to intrinsic resistance to MEK inhibitors in oncogenic *KRAS*-mutant cells[68].

3.4. *ERK inhibitors*

Compared with the progression and clinical application of RAF and MEK inhibitors, the development of ERK1/2 selective inhibitors lags far behind. This might be due to an earlier assumption that ERK is the only downstream target of MEK, and so no additive benefits were expected over MEK inhibitors[69]. However, discovery and development of ERK inhibitors has gained an increasing interest with the difficulties faced by RAF and MEK inhibitors, as well as with the deeper understanding of the MAPK pathway.

3.4.1. *The advantage of targeting ERK kinase*

Despite the exciting anti-tumor activities and survival benefits achieved by the approved RAF and MEK inhibitors, the unavoidable drug resistance has become the main challenge of designing and developing novel inhibitors targeting MAPK pathway[69]. The underlying mechanisms, which generally stem from cancer heterogeneity and genomic instability, are mostly related to the compensatory activation of upstream components. More extensive exploration of the biology of the MAPK pathway has elicited the proposition that targeting the downstream kinase ERK, as well as combining ERK inhibition with RAF and MEK inhibitions would be beneficial.

ERK sits at a unique position in the MAPK pathway, as the upstream molecule RAF has very few effectors besides MEK, which has no substrate other than ERK; and ERK is the only activator that is able to stimulate the wide variety downstream substrates[1]. ERK1/2 inhibitors can reverse the abnormal activation of MAPK pathway induced by upstream mutations including *RAS* mutations[61,70]. ERK is not only downstream of RAF, but also a negative inhibitor of RAF[23]. Furthermore, accumulating evidence suggests that subtle differences in the spatio-temporal activation of ERK generate variations in signaling outputs that regulate biological responses. Moreover, crosstalk between ERK and other pathways has been shown to be crucial for determining cell fate[18]. For example, a recently discovered oncogenic factor *O*-GlcNAc was linked to classical ERK signaling which is essential for the maintenance of the malignant phenotype of cancers[71]. All these have emphasized the potential benefits that can be gained by targeting ERK kinase in mutant MAPK pathway cancers.

More importantly, ERK inhibitors are able to overcome the acquired drug resistance induced by upstream kinases inhibitors (Fig. 4). Substantial studies have indicated that the reactivation of the MAPK pathway is a crucial event of acquired resistance of BRAF and MEK inhibitors[38,61,62]. The occurrence of acquired drug resistance to MAPK pathway inhibition involves a series of complicated mechanisms, which mainly include wild-type *BRAF* amplification, *BRAF-V600E* amplification and *MEK1/2* amplification or mutation[38,64]. Selective ERK inhibitors were reported to reverse acquired resistance to MEK inhibitors[70] as well as double drug resistance to BRAF and MEK inhibitors[40,64]. It is believed that ERK inhibitors may be less sensitive to resistance mechanisms than inhibitors of the upstream molecules in MAPK pathway. Thus, targeting ERK is considered to be more effective than

Table 2 Current status of ERK inhibitors[a].

Phase	Drug name	Organization	Indications
Biological Testing	FRI-20, ON-01060	Onconova, Temple University	Cancer
Biological Testing	VTX-11e	National Institutes of Health (NIH)	Cancer
Preclinical	25-OH-D3-3-BE, B3CD, bromoacetoxycalcidiol	Aphios, Boston University School of Medicine	Neuroblastoma; Cancer, prostate
Preclinical	FR-180204	AstellasPharma	Rheumatoid arthritis
Preclinical	AEZ-131, AEZS-131	AEternaZentaris	Cancer
Preclinical	AEZS-136	AEternaZentaris	Solid tumor
Preclinical	SCH-772984	Merck & Co.	Cancer
Preclinical	AZ-13767370	AstraZeneca	Cancer
Preclinical	BL-EI-001	Sichuan University, Tsinghua University, Shenyang Pharmaceutical University	Cancer
Phase I	LY-3214996	Eli Lilly	NSCLC, pancreatic cancer, CRC, melanoma
Phase I	LTT-462	Novartis	NSCLC, melanoma, ovarian cancer, NSCLC
Phase I	KO-947	Kura Oncology, Araxes Pharma	Cancer
Phase I (Terminated)	CC-90003	Celgene	Cancer
Phase I (Terminated)	GDC-0994, RG-7842	Genentech; Array BioPharma	Solid tumor, NSCLC, CRC, melanoma
Phase I	MK-8353, SCH900353	Merck Sharp & Dohme	CRC, NSCLC, melanoma
Phase I/IIa	BVD-523, Ulixertinib	Biomed Valley Discoveries, Vertex	Acute myeloid, solid tumor, melanoma

NSCLC, non-small cell lung cancer; CRC, colorectal cancer.
[a]Source from Thomson Reuters Integrity.

targeting BRAF or MEK in various forms of acquired resistance[64]. As such, combinational usage of ERK inhibitors and upstream inhibitors has become an important strategy to overcome acquired resistance and optimize therapeutic efficacy. For example, one of the selective ERK inhibitors, SCH772984, was found to re-sensitize tumor cells after the emergence of resistance to BRAF inhibitors or MEK inhibitors, offering the rational for combination of ERK inhibitors with inhibitors of BRAF or MEK[72]. Another study demonstrated that dual inhibition of MEK and ERK synergistically inhibited the emergence of resistance and overcome acquired resistance to MEK inhibitors[40]. Indeed, apart from the MAPK pathway *per se*, in many cases of drug resistance induced by upstream inhibitors, ERK inhibitors was discovered to be capable of retaining their ability to suppress the MAPK pathway and overcome drug resistance. It has been shown that acquired resistance to PLX4032 developed by mutually exclusive PDGFRβ (also known as PDGFRB) upregulation or NRAS mutations, is mediated by the reactivation of the MAPK pathway, and can be reversed by downstream inhibition[73,74].

3.4.2. *Current status of ERK inhibitors*

According to the data from Thomson Reuters Integrity (Table 2), only two ERK inhibitors that are BVD-523 and GDC0994, have reached clinical trial until very recently a few more inhibitors are reported. Considering the fact that ERK1 and ERK2 were founded three decades ago, the design and development of ERK inhibitors has lagged far behind compared with the upstream inhibitors.

BVD-523 (Ulixertinib, BioMed Valley) is an ATP competitive, kinase selective inhibitor. The IC_{50} against ERK1 and ERK2 are 300 and 40 pmol/L, respectively. BVD-523 showed its anti-proliferative activity in cell lines with activating *BRAF* mutations such as Colo205, as well as *KRAS* mutant colorectal and pancreatic cell lines. Importantly, BVD-523 is effective in several models that show intrinsic or acquired resistance to other MAPK pathway inhibitors. BVD-523 inhibits growth in wild-type cells and a RAF/MEK cross-resistant cell line bearing a *MEK1-Q56P* mutation with similar potency. Single-agent BVD-523 inhibits the growth of a patient-derived tumor xenograft harboring cross-resistance to dabrafenib, trametinib, and the combination treatment following clinical progression on a MEK inhibitor. BVD-523 was found to be efficacious in patient-derived xenografts resistant to vemurafenib[75]. Being consistent with its mechanism of action, strong pharmacodynamic effects were observed against p-ERK and downstream substrates. BVD-523 is currently the most advanced ERK inhibitor in clinic. BVD-523 is in the recruitment stage of two phase I/II clinical trials for solid tumors and hematologic malignancies, which have completed dose-escalation studies. It is also in phase I/II stage for acute myeloid leukemia (AML) or myelodysplastic syndromes. A phase I/IIa trial results recently released at the annual meeting of the American Society of Clinical Oncology (ASCO) in June, 2017 observed 2 patients with partial responses (3/27, 11%) during dose escalation and an additional 11 partial responses (13%) were observed during the expansion part of the trial, including 1 patient with melanoma resistant to BRAF/MEK inhibitors. Side effects are comparable to those seen with MEK inhibitors. BVD-523 received Food and Drug Adminstration (FDA) Fast Track designation in September 2015. Phase I clinical trials in pancreatic cancer in USA are underway as well.

GDC-0994 (RG-7842, Genentech/Array) is a very potent dual inhibitor of ERK1/2 (with IC_{50}'s of 1.2 and 0.3 nmol/L, respectively). GDC-0994 has shown promising combination activity with cobimetinib in *RAS*-mutated cancer cell lines and animal models. GDC-0994 is now in the recruiting stage of a dose escalation trial in patients with locally advanced or metastatic solid

tumors[76]. Combination of GDC-0994 and MEK inhibitor cobimetinib shows enhanced anti-tumor activity in *KRAS* and *BRAF* mutant tumor models[40].

SCH-772984 is a highly potent (ERK1/2 IC_{50} are 4 and 1 nmol/L, respectively) kinase-selective compound. Similar with VTX-11e, SCH-772984 was found to inhibit p-RSK, yet unlike other ATP competitive ERK inhibitors, it also showed a distinct ability to inhibit phosphorylation of the activation loop of ERK by MEK in A375 melanoma cells. It is noteworthy that SCH-772984 inhibits both the kinase activity of its target as well as prevents its phosphorylation by upstream effectors, analogous to the ability of RO5126766 to both inhibit MEK and prevent its phosphorylation by RAF[77]. In additional-cell based studies, SCH-7772984 was found to strongly inhibit the growth of a vemurafenib-resistant cell line derived from A375 cell line with an acquired *KRAS-G13D* mutation[78].

AEZS-136 is a highly potent and selective ATP-competitive ERK inhibitor, which can overcome the RAF inhibitor-induced paradoxical cell activation and acquired resistance to MEK inhibitors in tumor cells. Compared with common RAF inhibitors, AEZS-136 shows a more potent capability to prevent *BRAF* wild-type, *BRAF-V600E* mutant, *RAS* wild-type and *KRAS* mutant tumor cell line proliferation. AEZS-136 is efficacious in MEK inhibitor resistant HCT-116 and MDA-MB-231 cells which have been well characterized in terms of the *MEK-F129L* allosteric binding pocket mutation, as well as with varying degrees of *KRAS* amplification, and in cellular proliferation assays and MAPK pathway phosphorylation studies[79].

(*S*)-14k is an ATP competitive ERK inhibitor and an orally bioavailable agent which inhibits tumor growth in mouse xenograft models. On the basis of its *in vivo* efficacy and preliminary safety profiles, (*S*)-14k was selected for further preclinical evaluation[72]. (*S*)-14k (Array) and VTX-11e (Vertex) are both analogs with structural similarities to RG-7842. The binding mode of VTX-11e was determined by X-ray co-crystallography with ERK2 and involves key interactions between the hinge NH and main chain carbonyl of Met108 with the amino pyrimidine. Identified using structure-based drug design, VTX-11e was selected for more extensive profiling due to its superior potency against ERK2 (K_i <2 nmol/L) and high selectivity (>300-fold) against GSK3, CDK2, and Aurora A[80]. Despite the competitive position of this series from a timing standpoint, it does not appear that a member of the series has entered the clinic.

4. Perspective

MAPK cascades were discovered more than three decades ago and new functions are continuously revealed[50,55]. With the emergence of acquired drug resistance after treatment onset as well as the deeper appreciation that has been obtained in exploration of ERK kinase, there is a growing interest in targeting ERK kinase. The combination usage of ERK inhibitors with upstream inhibition may result in more desirable therapeutic benefits[40,41,64]. It is now clear that combination of different inhibitors of the same target, or drugs for different targets within the same pathway, can result in marked differences in effectiveness compared with single inhibitor, depending on tumor type and mutational status. For example, Moriceau et al.[39] has found that low concentration of ERK alone cannot reverse BRAF-inhibitor and MEK-inhibitor double resistance (DDR, double-drugs-resistance) unless co-targeting BRAF and MEK. High concentration of ERK inhibitors can inhibit the growth of DDR cells, but brings serious adverse effects. Of note, it also has been reported that BRAF inhibitor-resistant melanoma cells also often fail in response to ERK inhibition, which is explained by the ERK-dependent feedback loop to activate RAS and PI3K signaling. This study showed a broader targeting strategy that combining ERK and PI3K/mTOR inhibitors showed sufficient activity in tumors with BRAF inhibitor resistance[68]. Considering the breakthrough of immunotherapy, the combination of inhibition of MAPK kinases and immunotherapy may equip doctors with new weapons in cancer treatment one day[81]. Currently, ERK inhibition is still at its early stage in clinical studies. Clinical studies have not reported the occurrence of acquired resistance to ERK inhibitors. Nevertheless, it is important to note that the possible occurrence of ERK inhibitor resistance caused by ERK1/2 mutation prompts a new question on how to deal with this resistance[82].

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