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LETTERS

Exploring small-molecule inhibitors targeting MAPK pathway components: Focus on ERK, MEK1, and MEK2 kinases in cancer treatment

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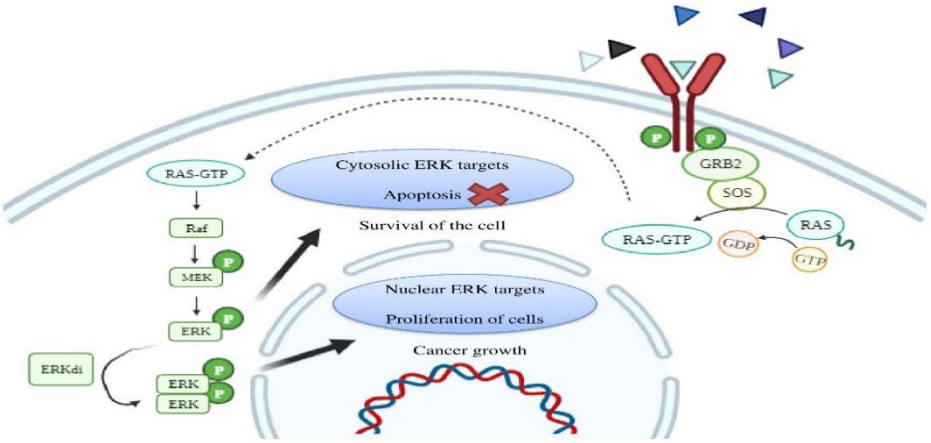
Review

ABSTRACT



Protein kinase cascades activate extracellular signal-regulated kinases (ERKs), or mitogen-activated protein kinases (MAPKs), which are involved in a variety of signal transduction pathways. This article will review the present state of MAPK pathway inhibitors, emphasizing the characteristics of tiny molecule blockers of the p38, MEK1, and MEK2 protein kinases. Many of these inhibitors have shown potential in experimental animal models of disease, and they are now being investigated in people for inflammatory and cancer diseases. Clinical trials are currently evaluating targeting a subset of cellular signaling cascades and signaling cascades that control pleiotropic cellular activity. These activities will have far-reaching consequences for managing a wide range of disorders. On the other hand, the Ras-Raf-MEK-ERK pathway is a clear therapeutic target because it is a standard downstream route for a range of critical growth factor tyrosine kinase receptors frequently changed or overexpressed in human malignancies. Several new medicines that target this route have been discovered and are currently being tested in clinical studies. BAY 43-9006 is one of the most intriguing new agents. Its ability to target Flt-3, c-Kit, and VEGFR-2, despite its initial development as a Raf kinase inhibitor, helps to explain its antiproliferative and antiangiogenic properties. This study will examine the ERK signaling pathway in both malignant and normal tissue, with an emphasis on new therapeutic approaches that target the ERK cascade at the Raf kinase level.

Keywords: MAP kinases, Small-molecule inhibitors, Inflammation, Cancer, ERK signaling pathway.

INTRODUCTION

The Ras-Raf-MEK-ERK pathway or the Ras-MAP kinase pathway is involved in several stages of the carcinogenesis process. This can be understood from the molecular framework regarding the study of cell signaling. This signaling pathway is being examined as a target for developing novel anticancer medications with promising efficacy with fewer side effects than the existing cytotoxic regimens. Mammals' central regulation of cell growth is mediated by the ERK pathway, which binds to a variety of cell surface tyrosine kinase receptors, including MET, HER-2, vascular EGFR (VEGFR), platelet-derived growth factor receptor (PDGFR), and epidermal growth factor receptor (EGFR). Extracellular ligands initiate signals.[1] These signals are relayed to the nucleus through a sequence of phosphorylation processes whereby start when Ras is activated. The next essential step in this pathway is Raf kinase, activating a class of serine-threonine kinases. Raf kinase triggers phosphorylation and activation of MEK1/2, which phosphorylates and stimulates ERK1/2. The phosphorylated ERK1/2 binds to numerous transcription factors. These RNA polymerases stimulate gene transcription, cytoskeletal alterations, cell proliferation, etc. Cell cycle activation-induced insensitivity to growth-inhibitory

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signals, telomerase induction leading to cell immortalisation, angiogenesis, growth factor independent proliferation and eluding apoptosis could all be elevated by fraud signaling through the ERK pathway. This suggests that the ERK pathway could be a promising site of action for new anticancer therapies. The intracellular protein serine or threonine kinases known as MAPKs are triggered by receptor tyrosine kinases and cytokine receptors, and heterotrimeric G proteins. The extracellular signal-regulated kinases, often known as MAPKs or ERKs, are classified as ERK1 and ERK2. Both ERKs are activated by protooncogene-encoded proteins that facilitate proliferation and a variety of extrinsic stimuli.[2] The Ras-MAP kinase pathway regulates crucial cellular signaling, activating kinases upon Ras stimulation. It impacts diverse cellular components such as the nucleus, cytoplasm, cytoskeleton, and cell membrane. Raf and MEK are prime targets for anticancer drugs due to their central role. Downstream, jun kinase and p38 families are activated by low molecular weight G-proteins, offering potential therapy targets. ERK1 and ERK2 are often overexpressed in solid tumors, driving cell proliferation via Ras-induced signaling.[3] ERK phosphorylates ternary complex factors, modulating mitogen-sensitive genes. Despite its complexity, pharmacological inhibitors target key proteins like ERK, Raf, MEK and Ras. Ras activates Raf-1 in a GTP-dependent manner, highlighting the pathway's intricate nature. Understanding these interactions is vital for effective anticancer therapies.[4]

The RAS kinase families, including c-Raf-1 and A-Raf, regulate proliferation, differentiation, and apoptosis. Constitutively active mutated Raf-1, preferentially activated by oncogenic H-Ras, contributes to cancer development, often independent of Ras signaling. Targeting Raf-1 shows promise in anticancer therapy, particularly in cells with aberrant growth factor signaling. Downstream, MEK serves as a viable target due to its phosphorylation of ERK1 and ERK2, critical in signal integration. MEK activation leads to cellular transformation, though not inherently oncogenic. MEK inhibitors like PD98059 motivate investigation into MEK as a potential anticancer drug target. Understanding the interplay between these kinases offers insights into developing effective therapies against cancer.[5]

These experiments revealed suppression of proliferation and numerous biological functions, including apoptosis, angiogenesis, etc as a result of MEK inhibition. MEK can thus act as a promisable target for pharmaceutical drug development against cancer. As a result, ERK, MEK and Raf-1 appear to be potential targets for anticancer drugs. Advanced pharmaceutical library screening for inhibitors of small molecules has undoubtedly resulted in several potential therapeutic candidates that target all phases of this process. For example, an assay of a cascade capable of detecting suppressors of c-Raf-1, MEK1 and ERK2 has been observed. It's critical to understand that these small molecule inhibitors' ultimate promise or variations may be defined by their four pharmacological characteristics as much as by the advantages of the kinase they target as we proceed with preclinical and clinical testing of them.[6] It is critical to remember that the Ras-MAP kinase pathway's component identification is probably incomplete. RKIP, a protein interacting with Raf-1, was discovered recently. This protein has been found to co-localize with Raf-1 and suppress Raf-1-triggered MEK's phosphorylation and activation. The binding of Raf-1 or MEK with RKIP is believed to separate complexes formed between Raf and MEK, interrupting MEK and downstream signaling. RKIP expression in tumour cells was discovered using a yeast two-hybrid system, although there is no particular significance of RKIP expression in tumour cells concerning signal transduction.[7] Moreover, unless we understand if RKIP expression is regulated negatively, it's ambiguous to boost its expression pharmacologically to inhibit tumour growth. Though on our present view on the role of RKIP, it is feasible that enhanced expression of RKIP can help tumor cells resist MAPK pathway inhibitors. Plenty of proteins have been identified recently that could help researchers develop new cancer medicines based on signal transduction targeting the MAP kinase pathway. A scaffold, Sur-8, also the kinase suppressor of Ras (KSR), promotes Raf-Ras interaction, which enhances Ras-MAP kinase signaling; it is believed to act as a protein scaffold for the Ras-MAPK pathway.[8] Another protein MP-1, which is noteworthy, has been observed to bind to MEK, which in turn activates MAPK. Moreover, a new ERK, ERK1b, which is found to be up regulated in Ras-transformed cells, is an alternative spliced form of ERK1. Gene expression regulated by transcription factors receive signals from cell surface receptors via the Ras/Raf/MEK/ERK pathway. Moreover, the path also governs several protein activities associated with apoptosis. In some malignancies, chromosomal translocations such as BCR-ABL, wild-type or mutated cytokine receptors including Flt-3, Kit, or FMS, or overexpression of wild-type or mutant receptors, such as EGFR, activate the pathway. By phosphorylation of molecules that regulate apoptosis after translation, like Bad, Bim, Mcl-1, caspase9 and, additionally controversially, Bcl-2, the Raf/MEK/ERK pathway has a significant control over apoptosis.[9]

Gene expression, apoptosis, differentiation, and cell cycle progression are all regulated by the Ras/Raf/MEK/ERK pathway. Raf phosphorylates apoptosis-related proteins without the aid of MEK/ERK. Its activity is influenced by interactions with other signaling pathways, namely alterations in upstream receptors such as B-Raf and Ras. Cancer cells become more resistant to chemotherapy when this pathway is activated. Since B-Raf mutations are common in many malignancies, targeting this route with medication is essential. Clinical trials are underway for several inhibitors that target downstream effectors, Ras, Raf, and MEK, indicating their potential for use as therapeutics.

NORMAL PHYSIOLOGY OF RAF AND MAPK PATHWAY IN PROLIFERATION

Proto-oncogenes are the initial step towards normal cellular function, functioning cooperatively within gene sets. MAPK modules, comprising three sequentially active protein kinases, regulate vital signal transduction pathways controlling cell differentiation, proliferation, and death across eukaryotes. Extracellular signals trigger each cascade, activating specific MAPKs and stimulating MAPK kinases (MAPKKs). These interactions, often mediated by small GTPases or downstream

protein kinases from cell surface receptors, activate MAPKK through phosphorylation. Activated MAPKKs, in turn, initiate MAPKs via dual phosphorylation of a conserved TxY motif. Once activated, MAPKs phosphorylate various substrates in the cytosol and nucleus, eliciting biological responses including protein function alterations and gene expression changes. MAPKs exhibit docking sites for MAPKKs and substrates, facilitating high-affinity interactions and specific downstream target recognition. Mammalian MAP kinases, such as ERKs, JNKs, and p38/SAPKs, exhibit distinct activation motifs and functional roles. ERK1/2, responsive to growth stimuli, are regulated by cell surface receptors like integrins, GPCRs, RTKs, and GTPases like Ras and Rap. MEK1/2 serve as MAPKKs for ERK1/2, while JNKs, activated by environmental stresses like heat and DNA damage, are regulated by various MAPKKs including members of the Raf family. Understanding these signaling cascades provides insights into cellular responses and offers therapeutic avenues targeting aberrant pathway activation in diseases like cancer.[13]

Cdc42 and Rac, specific GTPases belonging to the Rho family, signal to the JNK module, regulating apoptosis, inflammation, cytokine production, and metabolism. MAPKKs for JNK include MLK2, MLK3, ASK1, MEKK1, MEKK4, TAK1, and Tpl2, while MKK4 and MKK7 are MAPKKs. The p38 family, comprising p38a, p38b, p38g, and p38d, activated by environmental stressors and cytokines, regulates inflammation, necrosis, differentiation, and cell cycle. MKK3 and MKK6 serve as key MAPKKs for p38, alongside MLK2, MLK3, MEKKs, ASKs, TAK1, and TAO1/2 as upstream regulators. p38 substrates include MK2/3, PRAK, MSK1/2, and various transcription factors. Scaffold proteins like KSR, MP1, JIP1-4, and POSH facilitate MAPK signaling by localizing components, directing them to specific cellular locations or substrates, and regulating cascade timing. Ras, a small GTP-binding protein, acts upstream in pathways like Raf/MEK/ERK, PI3K/Akt, and RalEGF/Ral. Four Ras proteins—Ha-Ras, N-Ras, Ki-Ras 4A, and Ki-Ras 4B—exist, with Ki-Ras synthesized in two isoforms via alternative splicing. Ras proteins vary in their ability to activate Raf/MEK/ERK and PI3K/Akt pathways, with Ki-Ras being more potent in activating Raf/MEK/ERK than Ha-Ras. Understanding the intricacies of these signaling pathways provides insights into their roles in disease and cellular physiology, offering potential therapeutic targets for various conditions.[14]

Ha-Ras is a potent inducer of the PI3K/Akt pathway compared to Raf/MEK/ERK. Ras mutations are frequent in cancer, with Ki-Ras commonly mutated but N-Ras prevalent in certain cancer subtypes. Ras requires farnesylation or geranylgeranylation for membrane localization, with farnesylation preferred in Ha-Ras and geranylgeranylation in N-Ras and Ki-Ras. Palmitoylation, crucial for plasma membrane protein localization, occurs in both Ha-Ras and N-Ras, with Ha-Ras possessing two palmitoylation sites. These post-translational modifications offer therapeutic targets. Activation of Ras by the Shc/Grb2/SOS complex, triggered by cytokines or growth factors, switches Ras from GDP to GTP-bound active state. Active Ras recruits Raf to the membrane, initiating downstream signaling. Mutations at positions 12, 13, 59, and 61 are common in Ras mutations associated with human cancer, highlighting their significance as potential therapeutic targets. Understanding Ras signaling dynamics provides insights into cancer pathogenesis and therapeutic interventions.[15]

Post-translational modifications, including phosphorylation, render Raf constitutively active, bypassing ligand dependence. The mammalian Raf gene family comprises A-Raf, B-Raf, and Raf-1. Raf activation involves plasma membrane recruitment mediated by Ras, dimerization, and specific domain phosphorylation. Regulatory proteins like RKIP, BAG1, and Hsp90 modulate Raf activity. Raf-1 possesses multiple regulatory phosphorylation sites, with S43, S259, and S621 phosphorylated in the inactive state. Phosphatases transiently dephosphorylate S621 upon cellular stimulation, while S259 is targeted by phosphatases like PP2A. These dynamic modifications regulate Raf activity and its role in cellular signaling pathways.[16]

14-3-3 dissociates from Raf-1 upon phosphorylation at S338, Y340, and Y341, likely mediated by Src family kinases, activating Raf-1/MEK/ERK signaling. Src inhibitors like dasatinib, used in CML and other cancers, may inadvertently diminish this pathway's activity. A-Raf retains Src phosphorylation targets Y299 and Y300, while B-Raf substitutes them with aspartic acid (D492 and D493), enhancing basal activity. Ras and Src jointly activate Raf-1 and A-Raf, but B-Raf activation is Src-independent. B-Raf, with higher mutation rates in cancer, may simplify activation pathways. Dasatinib's effects on B-Raf signaling are complex due to potential B-Raf: Raf-1 heterodimer signaling. The role of Y340/Y341 phosphorylation in B-Raf: Raf-1 heterodimer activation is unclear. Ras promotes Raf-1 S338 phosphorylation, controlled by PAK. Additional Raf-1 phosphorylation sites include S43, S339, T491, S494, S497, S499, S619, and S621, possibly influencing activity. PKC can activate Raf-1 via phosphorylation at S497 and S499, although their necessity for Raf-1 activation is debated. Crosstalk between PKC and Raf/MEK/ERK pathways underscores the complexity of Raf regulation.[18]

Phosphorylation of the CR2 regulatory domain inhibits Raf activity, with S259 on Raf-1 phosphorylated by Akt and PKA, further inhibiting activity. B-Raf's kinase activity is suppressed by Akt or SGK phosphorylation at S364 and S428. Phosphorylated Raps bind 14-3-3, rendering them inactive. RKIP, a member of the PEBP family, inhibits downstream signaling by binding to Raf-1 or MEK/ERK. PKC phosphorylation of RKIP at S153 disrupts Raf-RKIP interaction. Hsp90 regulates active Raf, with inhibitors like geldanamycin promoting Raf degradation but affecting other targets like Src, EGFR, and Akt. Caspases also target Raf for degradation. The multifaceted regulation of Raf activity highlights its importance in cellular signaling and potential therapeutic interventions.[19]

In this instance, Raf is destroyed, and the cycle of kinases and phosphatases activating and inactivating Raf is irrevocably disrupted. After discovering that B-Raf is a substantially more potent MEK activator than Raf-1 and A-Raf, Raf-1's position in

the Raf/MEK/ERK signal transduction pathway has also been called into question. Several of Raf-1's "functions" are retained in Raf-1 knock-out mice, probably sustained through internal B-Raf. B-Raf is not just the principal MEK1 activator but is also implicated in Raf-1 activation, which is intriguing and contentious. B-Raf could be activated first, followed by Raf-1. However, B-Raf and Raf-1 may play different roles in signaling and apoptotic pathways inside the cell due to their other subcellular localisations.

Dimerization is crucial for Raf activation, with occasional B-Raf heterodimerization with Raf-1 for signal transduction. Complexities in the Raf/MEK/ERK cascade contribute to fine-tuning cellular responses. B-Raf:Raf-1 heterodimers have diverse substrate specificities and interactions, possibly influenced by kinase-deficient B-Raf mutants. MEK1, a dual-specific protein kinase, is phosphorylated on S residues within its catalytic domain, enhancing Raf activity. While all Raf family members can stimulate MEK, their potencies vary (B-Raf > Raf-1 > A-Raf). Activated MEK1 mutations reduce cytokine reliance and alter cell morphology. Ras and downstream effectors modulate expression of cell cycle regulators like p16Ink4a, p15Ink4b, and p21Cip1, inducing premature G1 arrest and aging. Dysregulated Raf/MEK/ERK pathway links to cell growth, cycle arrest, and apoptosis. Raf isoform expression influences cellular fate, with varied effects in different cells, including proliferation or arrest. Understanding these dynamics provides insights into cellular behavior and potential therapies.[21]

Overexpression of Raf isoforms in NIH-3T3 fibroblasts and FDC-P1 hematopoietic cells can induce cell growth arrest (B-Raf) or proliferation (A-Raf or Raf-1), depending on the isoform. The specific activity level of the Raf oncoprotein may influence these divergent outcomes. In NIH-3T3 cells, A-Raf upregulates Cdk2, Cdk4, cyclin D1, and cyclin E, while downregulating p27, promoting G1 to S phase transition. Conversely, B-Raf and Raf-1 increase p21Cip1, leading to G1 arrest. A-Raf, although a weak Raf kinase, induces cyclin expression and Cdk activity, promoting proliferation in FDC-P1 cells. Understanding the distinct roles of Raf isoforms sheds light on their contributions to cellular behavior and potential implications in cancer.[22]

On the other hand, ectopic manifestation of the much more powerful B-Raf caused apoptosis. The various consequences of A-Raf, B-Raf, and Raf-1 could explain the varied proliferative outcomes achieved with the three Raf genes. The roles of these three distinct Raf proteins aren't entirely known. Even though oncogenic Ras activates all three Raf proteins, they all target almost downstream molecules that are identical (MEK1 and MEK2) and employ conformational stabilisation, the same adaptor protein, both biochemical and biological features differ, and their roles are not always compensable. It's safe to assume that as we understand more about the complexities of these Raf compounds, further questions about their specificities and mechanisms of action on cell proliferation will emerge. Raf appears involved in several kinase cascades and downstream signal transduction factors that regulate apoptosis. Both Raf by itself and the Raf/MEK/ERK cascade affect significant molecules involved in apoptosis prevention in different ways. It's been known for a while that the Raf/MEK/ERK pathway phosphorylates Bad on S112, which results in its deactivation and eventual sequestration by 14-3-3 proteins. The subsequent formation of homodimers by Bcl-2 can trigger an anti-apoptotic reaction. Both the pro-apoptotic Bim protein and the Mcl-1 anti-apoptotic protein is phosphorylatable upon activation of the Raf/MEK/ERK cascade. Once phosphorylated, Bim is ubiquitinated and redirected to the proteasome, disassociating from Bcl-2, Bcl-XL, and Mcl-1. Bcl-2, Bcl-XL and Mcl-1 can then attach to Bax, preventing it from activating and forming homodimers with Bax. Consequently, apoptosis is prevented. Bim ubiquitination and subsequent proteasomal breakdown may result from ERK phosphorylation of Bim on S69. Akt can also phosphorylate Bim at S87, reducing Bim's apoptotic potential and increasing 14-3-3 protein binding. On the other hand, JNK-mediated phosphorylation of Bim at S65 can cause apoptosis by stimulating Bax:Bax interactions, resulting in death. JNK also phosphorylates members of the 14-3-3 family, allowing Bax to be translocated from the cytosol to the mitochondrial membrane inducing apoptosis. The exact residues on which Bim is phosphorylated, clearly impact if a cell triggers apoptosis or survives. The Raf/MEK/ERK pathway has recently been shown to phosphorylate caspase 9 on residue T125, resulting in its inactivation. It's worth noting that the Akt pathway phosphorylates both Bad and caspase 9, this implies that the PI3K/Akt and Raf/MEK/ERK pathways work together to prevent apoptosis.[23] The phosphorylation site of caspase 9 by Akt is not evolutionarily conserved, raising questions about this event's significance.

The Raf/MEK/ERK cascade also phosphorylates Bcl-2 during particular residues in the loop region, which is connected to increased anti-apoptotic properties. As previously stated, raf-1 acts at the mitochondrial membrane in a MEK and ERK-independent manner by phosphorylating Bad, which causes it to detach from the mitochondrial membrane. According to recent research, RAF-1 converses using mammalian sterile 20-like kinase (MST-2) and prevents it from dimerising and activating. Pro-apoptotic drugs like staurosporine and Fas ligand activate MST-2, which is a kinase. MST-2 is bound by Raf-1 but not by B-Raf. Apoptosis sensitivity was abolished when MST-2 was removed from Raf-1 cells. MST-2 overexpression increased apoptosis sensitivity. MST-2 was assumed to be controlled by Raf-1 encasing it in an inactive combination. This Raf-1:MST-2 complex is devoid of MEK and downstream ERK. Raf-1 can potentially prevent apoptosis by interacting with ASK1. ASK1 is a universal apoptotic mediator triggered by cytotoxic stressors such as TNF, Fas, and ROS. ASK1 appears to be involved in stimulating the JNK and p38 MAP kinases. This is an entirely separate Raf-1 interaction from MEK and ERK. Growth factor expression, which often has autocrine effects, is a common characteristic of Raf-transformed cells. NIH-3T3 cells that have experienced Raf activation release heparin-binding epidermal growth factor (hbEGF). The autocrine growth factor granulocyte macrophage-colony stimulating factor (GM-CSF) is known to be expressed by hematopoietic cells that have activated Raf genes. B cells affected by Kaposi's sarcoma express high quantities of

B-Raf. High quantities of B-Raf are also produced by vascular endothelial growth factor (VEGF). B-Raf has recently been discovered to boost Kaposi's Sarcoma Virus infectivity. B-Raf's ability to stimulate VEGF expression is one mechanism accountable for its potential to promote viral infection. In the promoter regions of several growth factor genes, there are interacting locations where the Raf/MEK/ERK pathway phosphorylates transcription factors. As a result, an uncontrolled autocrine loop could be created by Raf expression that stimulates cell proliferation indefinitely. On the other hand, Raf-induced VEGF production has the potential to enhance angiogenesis. Since the expression of growth factors has been linked to medication resistance and apoptosis, the expression of growth factors triggered by Raf will aid in avoiding apoptosis and chemotherapeutic drug resistance.[24]

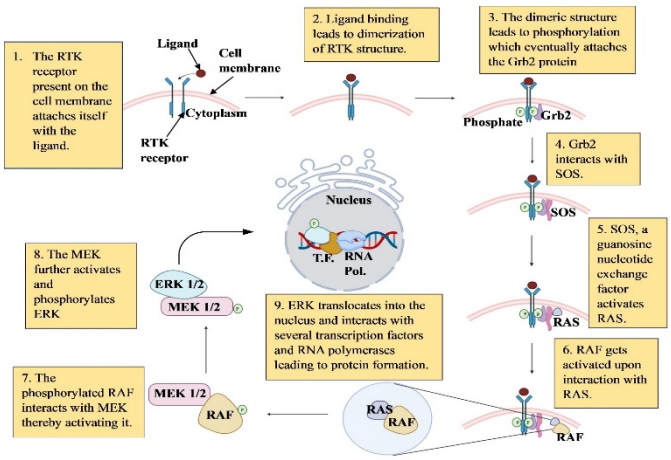


Figure 1. The above schematic diagram shows the RAF/MEK/ERK pathway overview.

Receptor tyrosine kinase (RTK), growth factor receptor-bound protein 2 (Grb2), fast-acting guanine nucleotide exchange factor (SOS), mitogen-activated protein kinase (MAPK) (also known as RAS-MAPK), rapidly accelerated fibrosarcoma (RAF), mitogen-activated protein kinase (MEK), extracellular signal-regulated kinase (ERK), and transcription factor (TF) are the symbols shown in the above diagram. As a signaling molecule, MAPK functions as a growth factor for the cell and aids in gene expression, apoptosis, and cell proliferation. This is the mechanism by which the RAS-MAPK pathway's downstream cascade occurs.

Altered mechanism in malignant pathology. About 30% of human cancers are caused by mutations that amplify the Ras proto-oncogene and activate it, resulting in increased Ras proteins. A B-Raf mutation has been found in roughly 7% of cancer cases. But if more and more tumors are examined for B-Raf mutations, this frequency might change. Recent research has shown that mutant Raf-1 alleles are frequently linked to therapy-induced acute myelogenous leukemia (t-AML).[25] Individuals with breast cancer who had treatment developed this kind of leukemia. They found Raf-1 genes, which underwent mutations and were inherited by subsequent generations. Therefore, these mutations in leukaemia didn't occur spontaneously, but they could be vulnerable to t-AML induction in these Austrian patients with breast cancer. One belief regarding Raf oncogenes was seldom mutated in human cancers for a couple of years. Thus, researchers concentrated more on Ras mutations, which might regulate the PI3K/Akt and Raf/MEK/ERK pathways.[26] Furthermore, just lately, it has been discovered that B-Raf mutations is frequent in cancers such as melanoma (27–70%), papillary thyroid carcinoma (36–53%), colorectal cancer (5–22%), and ovarian cancer (30%)—factors leading to B-Raf mutations in melanoma patients rather than Raf-1 or A-Raf mutations are unclear. Because of the mechanism of B-Raf activation, selecting B-Raf mutations may be easier than selecting Raf-1 or A-Raf mutations. As mentioned, two genetic changes are needed to activate Raf-1 or A-Raf, whereas only one genetic mutation is needed to start B-Raf. Recently, it was suggested that the structural properties B-Raf, Raf-1, and A-Raf could affect the possibility of triggering mutations occurring at these compounds, allowing carcinogenic versions to be selected.[27] These predictions were made after the crystal structure of B-Raf was discovered. A catalytic cleft separates the large and tiny lobes that are thought to be present in B-Raf, similar to many other enzymes. B-structural Raf's and catalytic domains, as well as the size and location of the tiny lobe, may all play an aspect of its ability to be brought about by triggering mutations. On the other hand, the exact alterations in A-Raf and Raf-1 aren't expected in resulting minor lobe stabilization, which inhibits mutated A-Raf and Raf-1 selection, leading to the activation of oncogenes. For a long time, the interaction between Hsp90 and Raf-1 is known. Raf-1, A-Raf and B-Raf that have been activated, may be stabilized by Hsp90. The role of Hsp90 in choosing the activated mutant Rafs is intriguing but highly speculative. The most prevalent B-Raf mutation is at nucleotide 600, where a change causes valine to become glutamic acid (V600E). 90% of B-Raf mutations in thyroid and melanoma tumors are caused by the mutant protein. It has been hypothesized that cells that produce high levels of B-Raf as a result of hormone stimulation will eventually develop mutant B-Raf. Some hormonal signaling events cause an increase in intracellular cAMP, which causes activation and proliferation of B-Raf. Thyrocytes and melanocytes are two cell types with high B-Raf expression because the right hormones frequently trigger them. Furthermore, B-Raf is regarded as the most critical kinase in the Ras/MEK/ERK pathway; mutations in B-Raf activate ERK and MEK in specific models. Mutations in B-Raf are thought as the beginning processes in particular cells, but not enough to cause full-blown neoplastic transformation.[28]

Certain mutations in B-Raf (e.g., V600E) and Ras may induce cell cycle arrest by hyperactivating the Raf/MEK/ERK pathway. However, some B-Raf mutations require both Ras and B-Raf mutations for transformation, leading to reduced B-Raf activity. Predominantly occurring at residue 600, mutations like V600E activate B-Raf, ERK, and MEK. Raf-1 signaling compensates for B-Raf mutations, potentially through heterodimerization. Alternatively, mutations like D593V may activate alternative pathways. Over 50% of acute lymphocytic leukemias (ALL) and

acute myeloid leukemias (AML) exhibit constitutive Raf/MEK/ERK activation, indicating potential therapeutic targets. The PI3K/Akt pathway, activated by Ras interaction or receptor tyrosine kinase binding, induces PIP3 formation, facilitating Akt activation via PDK1. Phosphorylation (Figure 2).

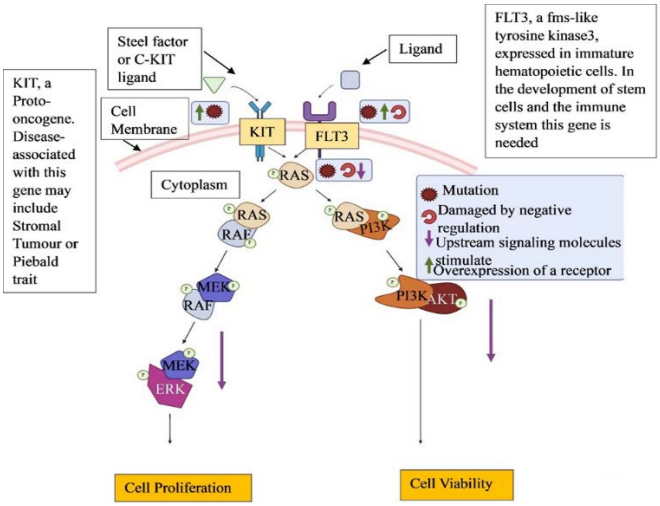


Figure 2. Altered molecular cascades in malignancies.

RAS ONCOGENE FAMILY: MUTATIONAL PATTERNS, DISTRIBUTION IN HUMAN CANCERS, AND PATHOLOGICAL SIGNIFICANCE

Over the last three decades, the Ras oncogene family has been intensively explored, with over 40,000 research papers published depending on the topic. Ras proteins are essential to several pathological processes, including cancers and physiological pathways that control cellular proliferation, differentiation, and death. This helps to explain why 200–300 publications are published in the scientific literature each month. About 30 years ago or more, the first discovered oncogenes in the tumour types of humans were H-Ras, N-Ras and K-Ras oncogenes, which are the pioneers of the Ras gene superfamily, now past 150 different cellular members. The family of Ras GTPases is a critical participant in many signaling pathways, linking a wide range of upstream signals to a wide diversity of downstream effector pathways, which determines various cellular outcomes, such as cell cycle progression, cytoskeletal changes, migration, senescence and apoptosis.[32] The interaction of these signalling pathways with others controlled by multiple groups of signalling molecules leads to molecular connections where balance is crucial in defining the cell's outcome of cellular events. The ambiguity of each of these activities that control cellular life represents those complications that must be resolved when these networks are interrupted in pathological disorders, highlighting the significance of examining them to strategies the therapeutic action that can restore a healthy signalling balance to cells. The Ras-Raf-MAPK pathway within the cellular signalling networks involves H-Ras, N-Ras or K-Ras, vital to eukaryotic cells' existence, differentiation, and proliferation. The increasing number of clinical disorders associated with anomalies in some of this pathway's components highlights the pathway's evolutionary relevance and importance. Molecular modifications of several other elements of the signalling system, for example, B-Raf EGFR, and NF-1, were observed in the promoting various malignancies, along with frequent mutations of the Ras genes, detected around 3 decades ago. Experiments have revealed that mutations of different signalling cascade components are generally exclusive in most cases, such as in the case of the B-RAF and RAS oncogenes in metastatic melanomas. Moreover, several elements of this pathway may experience concurrent molecular changes in a few instances. This is relevant in solid tumours because the occurrence of K-Ras mutations and concurrent overexpression of EGFR-related genes predicts responsiveness to novel EGFR-targeting drug therapies. N-Ras, K-Ras, or H-Ras gene mutations are three of the main reasons why cancer occurs in humans.[33]

Over the last three decades, numerous investigations have discovered two vulnerable areas for Ras oncogenic mutations, positioned near the highly conserved coding sequences containing codons 12 and 61, respectively. During this time, various databases were produced to house all of the information gathered about the existence of certain Ras gene mutations in multiple types of human malignancies. The Sanger Centre now maintains and upgrades a centralised record containing information on the type and incidence of Ras mutations in diverse human cancers of all human cancers examined, around 30% are identified to have a mutation in one of the classical Ras genes. Surprisingly, the K-Ras locus is mostly affected during oncogenic mutations, where K-Ras mutations are present in 25-30% of all tumour specimens tested. K-Ras mutations, along with their enormous rate, arise predominantly during the starting phase of tumour growth, which implies a pivotal function of K-Ras in human carcinogenesis. In contrast, the frequencies of mutated oncogenes detected in the H-Ras and N-Ras families are substantially low (3% and 8% of the tested samples).[34]

Development of regular mice does not require N-Ras or H-Ras; instead, it requires K-Ras, which is indicated by its compatibility with higher relevance towards the physiology shown by knockout mice strain phenotypes and its primary role in pathological tumour growth. After evaluating enormous sets of tumour specimens collected over the last 30 years, it has been concluded that distinct mutated Ras isoforms (although not bi-univocal) are associated with specific types of malignancies. In many cases of pancreatic ductal adenocarcinomas and lung and colon tumours, K-Ras mutations are found, whereas they are infrequent in the case of bladder tumours. Instead, H-Ras mutation is most often. Haematological malignancies and malignant melanomas have shown a prevalence of N-Ras mutations, with the rare existence of K-Ras and H-Ras mutations. Mutation in the K-Ras gene is usually observed in adenocarcinomas and solid tumours; in contrast, a mutation in the N-Ras gene is the most common in leukemia, thyroid carcinomas or malignant melanoma. Oncogenic mutations are localised in two foci (around codons 12 and 61) of all Ras family members' primary nucleotide sequences. Along the K-Ras locus, the mutation incidence at the biological significance of any different

mutation is hardly known. According to a recent study, exon 4 mutations may ensure a better prediction. In colorectal cancer (L19F and T20A), another study describes a unique group of transforming mutations that target codons 19 and 20.35 Mutations in N-Ras genes, in human malignancies follow a distinct pattern of distribution, having a maximum incidence of mutation observed at Q61 (nearly 60% of total mutation of N-Ras gene) and fewer percentages found at G12 (24.4%) and G13 (12.7%). Ultimately, mutation of the H-Ras gene follows its pattern, having the highest incidence of mutations found in codon 12 (almost 54%), codon 61 (nearly 34.5%), and codon 13 (almost 9%).[36] Though various pathways could activate Ras in vitro, mutations of the oncogenes seem to be practically the unique pathway that links Ras genes to human tumour growth in vivo. Despite some early findings regarding the amplification of N-Ras 33 or K-Ras 31, 32 in specific cell lines and tumours, most experimental data demonstrate that amplification of Ras is not a typical occurrence in cancer. Additionally, a current study examining Ras overexpression in colon cancer found no link between it and prognosis, implying that Ras overexpression is not a reliable predictor.[37] Ras amplification is unusual in malignancies, which may be explained by recent studies showing that the proportionate percentages of expressed H-Ras, N-Ras, and K-Ras proteins are almost constant in many tissues and cells investigated, regardless of whether they are tumoral or normal (Figure 3).

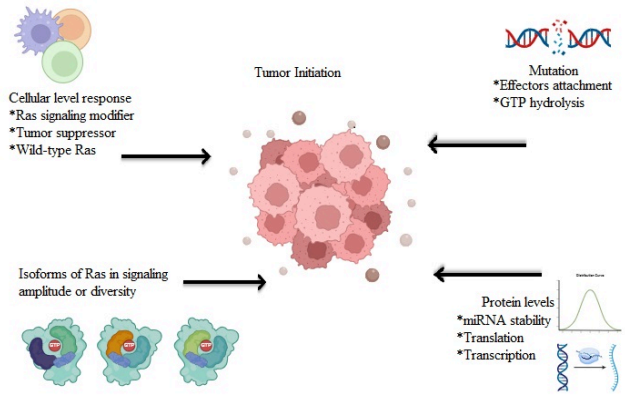


Figure 3. Mutational landscape and clinical relevance of the Ras oncogene family in human cancers.

RAF KINASES IN CANCER: MULTIFACETED ROLES IN TUMORIGENESIS AND STRESS RESPONSES

10 years ago, they mentioned in a legendary review that they identified six hallmarks of cancer, which describe a cancer cell's acquired cell autonomous abilities. Other properties of cancerous cells on environmental interaction, like escaping the immune system and the cancer stress traits and genomic instability, have recently been added to the list. The contribution of Raf and others will be highlighted in the next section. The auto-inhibitory N-terminal domain gets deleted consequent to the changes, leading to constitutive RAF activation. In prostate, gastric carcinoma, and melanoma, chromosomal translocations resulting in transcripts of gene fusion, including the C-terminal kinase domain of C-RAF or B-RAF, have low occurrence. In human prostate cells, both fusion proteins boosted migration, anchorage-independent growth, and MEK/ERK-dependent cell proliferation in NIH 3T3 cells; however, B-RAF fusion protein expression resulted in tumour formation in nude mice, but C-RAF fusion protein expression did not do so, stating crucial signaling differences between the B-RAF and C-RAF fusion proteins. The reciprocal fusion C-RAF, which contains the C-RAF regulatory domain was also found in prostate cancer; however, this protein has not yet been thoroughly explored. Furthermore, RAF has been linked to generating genomic instability and being a target of chromosomal rearrangements. So far, two mutation types are linked to enhanced genomic instability: B-RAFV600E being the most common stimulating mutation in melanoma and a few other carcinomas, it causes genomic instability in a thyroid cell line; besides that, expression of B-RafD594A, which is a B-Raf mutant having disabled MEK-kinase activity, enhances aneuploidy in mouse splenocytes and embryonic fibroblasts following C-Raf dependent and MEK independent manner. C-Raf, however, has been linked to chromosomal instability, perhaps in an indirect way. C-Raf and RKIP, the Raf antagonists usually lacking in breast, prostate, and melanoma malignancies, must be balanced to ensure chromosome segregation integrity. Aurora-B kinase activity is reduced when RKIP is less, or C-Raf is overexpressed, which allows the cells to escape the spindle assembly checkpoint and potentiate genetic instability.[38]

In the transformation process, being self-sufficient in signals promoting proliferation is essential. A cell type in healthy tissue synthesises soluble mitogenic growth factors which induces adjacent cell proliferation. Most cancerous cells can manufacture their growth factors and respond to them, creating positive feedback signaling circuit (autocrine stimulation), allowing them to function independently of their surrounding tissue. Growth factor production, overexpression of growth factor receptors and changes in downstream signaling pathways are all examples of these proliferative signals. The former two alterations may lead to Raf activation, even though the degree of dependence of the resultant proliferation may differ. Aside from Raf mutational activation, the straightforward link in signaling components is between the RAS gene family and RAF, which are active in 33% of human malignancies and are notable of epithelial origin. Numerous RAF mutations have been identified as influencing tumour progression. B-RAFV600E, the most common B-RAF mutation, induces constitutive kinase activation and resistance to adverse feedback pathways.[39]

B-RAF mutations that activate the wild-type C-RAF in the presence of a heterodimer, which can trigger the MEK/ERK pathway, have been identified. C-RAF mutations are exceedingly uncommon, but overexpression has appeared in several of human malignancies, primarily hepatocellular carcinoma and squamous cell carcinoma of the neck and head. C-Raf overexpression is thought to be a marker for early tumours in human lung adenocarcinomas. In human glioblastomas, increased kinase activity and enhanced expression of C-RAF and B-RAF have

been detected, and a constitutively mutated active C-RAF leads to the production of gliomas in mice. These results indicate that most Raf mutations cause proliferation by triggering the MEK/ERK pathway. Apart from the noteworthy function of B-RAF oncogenic mutants, endogenous, wild-type B-RAF stimulates the activation and growth of ERK in uveal melanoma cells devoid of RAS/RAF mutations; nevertheless, C-RAF, not B-RAF, is required for these procedures when mutated KRAS in non-small-cell-lung-cancer cell lines or mutated NRAS in melanoma cell lines are downstream.[40]

Autocrine/paracrine factors emerging from activation of ERK seem to impact the cells self-sufficient to harbour activated Raf mutations, producing a result-oriented loop and enhancing the simultaneous activation of concurrent proliferative processes, as per the experiments using in vivo and cultured cells. In addition to producing proliferative signals through cell-autonomous or paracrine means, malignant cells must to develop resistance to antiproliferative cues governing tissue equilibrium. The transforming growth factor-b (TGF-b) mainly induces the antiproliferative signals. The TGF-b receptor is downregulated or mutated in many cancers, and its substrates downstream to it, p15INK4B, SMAD4 and the retinoblastoma protein Rb are inactivated. TGF-b production is induced when Raf and ERK are activated. Yet, cells are protected from differentiation and apoptosis, allowing them to take advantage of the pro-tumorigenic effects of this cytokine, such as immunological suppression, radio resistance, invasiveness and proliferation. Rb also gets phosphorylated on its interaction with CRAF. This interaction leads to the formation of the Rb-CRAF complex, which promotes proliferation, enhancing E2F1-dependent transcription activation and, therefore, counteracts Rb's antiproliferative action.[41]

Differentiation is induced as a potent barrier to proliferative signals. The early progenitor cells or initial stem cells that give birth to tumours can be used in therapy if they have not lost their sensitivity to differentiation impulses. A notable example of this is the transformation in leukaemia treatment with the introduction of the combination of separation treatment and chemotherapy. A recent discovery suggests endogenous C-RAF is required to keep Ras-driven epidermal malignancies undifferentiated. Agitation of cytoskeleton-based kinase ROK-α triggers MEK/ERK-independent stimulation of a differentiation programme, conditional excision of C-RAF causes quick retroversion of existing malignancies. The possibility of differentiation (co)therapy in solid tumours is proved in these findings that demonstrate the sensitivity of Ras-driven tumours to non-oncogenic C-RAF.[42]

Recent studies have demonstrated the importance of Raf in maintaining an undifferentiated state by establishing that C-RAF amplification induces ERK-dependent b-catenin activation, as well as the generation of breast tumour-initiating cells in culture and carcinogenesis in xenografts. As previously demonstrated, full-length RAF or the truncated catalytic domain upregulation in cultured cells in vivo activates the ERK pathway. Potent pathway activation is linked to senescence development in both situations, and senescence must be prevented before hyper-proliferation can happen. Senescence is hence the Ras/Raf/Erk pathway's weak point. Reducing B-RafV600E mutant activity directly to a level that does not encourage senescence is one possible solution. In this case, it has been shown that endogenous C-RAF limits the activity of B-RafV600E when it forms a heterodimer, and it is also possible that Akt3 phosphorylates BRAF's negative-regulatory residues, which lowers the protein. Senescence is typically compromised when cooperative proto-oncogenes like c-Myc or Rac1b are expressed, or when tumour suppressors like p16INK4a, p19ARF, p53, or PTEN are muted.[43]

On the contrary, activation of RAF does not promote bypassing of senescence; instead, it plays several roles in resisting apoptosis, some of which are isoform-specific. Apoptosis results from the ERK pathway due to the modulation of the activity of BCL-2 family constituents downstream of active Raf and Ras and other oncogenes. Moreover, MEK-independent prosurvival mechanisms for C-Raf have been postulated, like MEKK1, the nuclear factor-kB pathway activation, and the BH3-only BCL-2 family member BAD inactivation. Establishing the association between the BCL-2 family and C-RAF, elimination of BCL-2 prevents the formation of lung adenomas caused by the abbreviated, carcinogenic variant of C-RAF.[44]

Moreover, native C-RAF inhibits cell death without the need for kinases by attaching to and blocking the stress-associated mitochondrial kinase ASK-1 and the homolog of Drosophila's Hippo, MST-2 kinase. Additionally, it regulates Fas trafficking by means of its association with the cytoskeleton-based kinase ROK-α. Any of these processes may encourage cancer by providing a survival advantage, however their importance in this context has not yet been established in vivo. Unlimited replicative capacity is possible by preventing telomere shortening, which results in a damage reaction on DNA that is carried out by p53 and p21, and eventually senescence. This telomere attrition-induced senescence contrasts from the rapid barrier to proliferation induced by an oncogene, which is seen in B-RAFV600E-expressing premalignant nevi. Telomerase upregulation, a reverse transcriptase that helps restore telomerase repeats at the end of each cell division, allows 85-90% of all cancerous cells to avoid telomerase degradation. Activated ERK targets Ets transcription factors, which enhances transcription. After oncogenic growth factor receptors, the telomerase catalytic subunit gene is located, and Raf and Ras potentially counteract telomere shortening and strengthening the mutated cells' replicative power. Solid tumours that develop larger than 3 cubic millimetres require continuous angiogenesis.[45]

Tumour cells can direct the angiogenic signals towards angiogenic starting signals such as fibroblast growth factor-1 and 2 and vascular endothelial growth factor or VEGF while suppressing prohibitor stimulations (thrombospondin-1 and interferon-b). A study was done in vivo in mice to understand how angiogenesis is affected by Raf, using a C-RAF construct devoid of the kinase that is introduced into the vasculature associated with the tumour. Apoptosis was induced by the kinase-dead protein in the endothelium and in tumour cells, resulting in tumour suppression. C-RAF is capable of enhancing the lifespan

of endothelial cells via MEK-dependent or independent pathways, which includes suppression of ASK-1. Moreover, suppression of both micro-vessels associated with tumour and tumour xenograft is observed when the association of C-RAF and Rb is disrupted. As a result, numerous C-Raf-dependent pathways may play a role in angiogenesis. B-RAFV600E, on the contrary, requires MEK to induce angiogenesis, which involves the production of VEGF and hypoxia-inducible factor-1a. In a mouse model having cancerous pancreatic islet caused by the inactivity of tumour suppressor genes p53 and Rb, conditional deletion of endogenous B-Raf inhibits the angiogenic transition.[46]

Despite low ERK activation, B-Raf-deficient tumour cells usually grow. However, they produce low levels of angiogenesis-promoting molecules, TGFb and VEGF, which results in low density of blood vessels along with tumour proliferation and a delay in tumour growth. Metastasis and tissue invasion are influenced by all other characteristics gained amidst tumour growth, along with alteration in the protein binding cells to their surroundings. The cancerous cells tend to invade and colonise healthy tissue is influenced by altered cell-cell adhesion molecules' expression and/or integrins' unique binding properties, along with the overexpression of extracellular proteases and its activation. The ability of Raf to impact invasion on multiple levels has been observed. To begin with, Raf on activation enhances TGFB creation. This growth factor enhances invasion and metastasis, along with the transition of epithelial mesenchyme which occurs before invasion as a response to this factor. Secondly, B-Raf and C-Raf both play critical although opposing functions in cell migration and contractility: B-Raf promotes Rho-dependent contraction and prohibits migration in an ERK-dependent way and on the other hand C-Raf suppresses the Rho effector ROK-α.[47]

Moreover, B-RAFV600E/MEK/ERK promotes melanoma cell invasion and metastasis by overexpressing numerous proteins involved in motility and supporting integrin signalling. B-RAFV600E/MEK/ERK can also enhance contractility and invasion of melanoma cells by suppressing the gene that codes for cGMP-specific phosphodiesterase, PDE5A. The increased cGMP pool increases intracellular calcium ions and, as a result, greater contractility, which enhances the rounded, bleb-associated mode required for mobility at the time of invasion. PDE5A expression was reduced in patient material generated from metastases compared to primary tumours. ERK is presumably triggered in both cases; watching to what degree further regulation of PDE5A expression occurs and what further events lead to entire metastatic inhibition would be fascinating. Changes in tissue architecture arean option here, as they can have powerful anti-proliferative and anti-invasion effects. In C-RAF-driven lung adenomas, for example, cadherin-based cell–cell adhesion effectively inhibits tumour proliferation, angiogenesis, and metastasis.[48]

Matrix metalloproteases, often upregulated in tumors, influence tissue architecture. Overexpression of matrix metalloprotease-9 via the Raf/MEK/ERK pathway disrupts breast tissue polarity and promotes proliferation in 3-D cultures. RKIP, a Raf inhibitor, suppresses metastasis by inhibiting the Raf/MEK/ERK pathway. RKIP also facilitates let-7 miRNA processing, inhibiting the chromatin-remodeling factor HMGA2 and metastasis-promoting gene expression. BRAFV600E aids tumor cell extravasation by inducing interleukin-8 production, attracting polymorphonuclear leukocytes to facilitate endothelium barrier traversal.[49]

Tumours can resist detection and eradication by the immune system by avoiding immune monitoring. Cancers dodge detection by choosing cancer cells that are not immunogenic, like those with down-regulated molecules expressing human leukocyte antigen class-I and/or are resilient to cytotoxic T-lymphocyte-induced death (immunoselection). On the other hand, tumours can suppress immune cells in various ways, resulting in an immune-privileged habitat (immunosuppression). B-RAFFV600E, for example, helps in the evasion of the immune with the help of MEK/ERK-dependent reduction human leukocyte antigen class-I molecules, antigen-specific T-lymphocytes (immunoselection), and/or in the production of melanoma differentiation antigens, and enables immunosuppression by activating the cytokines, interleukin-10 and interleukin-6.[50]

Treatment using B-RAF-specific inhibitors, unlike MEK inhibitors, does not impair T-lymphocyte function, generating optimism that such inhibitors could circumvent immune evasion. The above-mentioned characteristic events come at a high cost in terms of stress for tumours. Cancerous cells manifest five distinctive stress phenotypes: first, the shortening of telomeres and replication stress induced by oncogene activation leads to damaged DNA, compounded by mutations in DNA repair mechanisms, thereby exacerbating DNA damage in the context of RAF kinase involvement in cancer. Second, chromosomal instability triggers mitotic stress within these cells. Third, the accumulation of misfolded proteins precipitates proteotoxic stress. Fourth, the build-up of reactive oxygen species generates oxidative stress within tumour cells. Fifth, relying on aerobic glycolysis for ATP synthesis induces metabolic stress, collectively constituting a complex spectrum of stress responses observed in cancerous cells. What role does RAF play in coping with stress? Raf and Ras, on activation, stimulate the MDM2 gene expression in response to genotoxic stress, leading to p53 degradation; diminished apoptosis associated with the p53 gene, leading to damaged DNA, occurs in those cells that lack p19ARF, the Mdm2 inhibitor. The expression of the cyclooxygenase-2 gene mediated by ERK, which reduces apoptosis induced by genotoxic stress, is mediated by HB-EGF activation of Raf or Ras downstream of p53. Another factor for growth, the Scatter Factor (hepatocyte growth factor) which protects malignancies from genotoxicity, activates nuclear factor-kB to indicate survival.[51]

Furthermore, it has been reported that the interaction between ATM and RAF/ERK, on stimulation by radiation, enhances homologous recombination repair, consistent with prior results linking oncogenic Raf to tumour radio resistance. Ultimately, treatment with a Chk1 inhibitor causes DNA damage in many myeloma cells, but the RAF/MEK/ERK pathway protects them. These data reveal that inhibitors of RAF and MEK could be used

in cancer therapy alongside cytostatic medicines or radiation. RAF has numerous links with oxidative stress: Stimulated Raf/MEK/ERK inhibits oxidative stress emergence growth factor-depleted cells; however, geldanamycin derivatives, a chemotherapy drug that prevents HSP90 from acting as a chaperone, also promote degradation of their client proteins, as well as RAF and can suppress B-RAFV600E. Ultimately, activation of Raf and Ras oncogenes, as well as the small-molecule medication erastin, have recently been linked, which has implications for oncogene-selective therapy.[52]

Through the stimulation of mitochondrial voltage-dependent ion channels, also known as voltage-dependent anion channels, or VDACs, Erastian encourages oxidative cell death and mitochondrial malfunction. Activation of Ras and B-Raf increased the drug's lethality by promoting the expression of VDACs in a series of cancer cell lines from various origins. As a result, VDAC expression in RAS- and RAF-driven malignancies may constitute a targetable weak spot. Metabolic stress, particularly a shortage of nutrients, causes cancer cells to circumvent several metabolic checkpoints and continue growing in the harsh conditions prevalent in the tumour microenvironment. Melanoma cells expressing B-RAFV600E seem to handle this challenge by phosphorylating LKB1, both the energy sensor and the tumour suppressor, via ERK. Protein synthesis is restricted by this phosphorylation, which is facilitated by a physical complex containing B-Raf V600E. It also prevents LKB1-mediated activation of AMP-activated protein kinase. Under metabolic stress, the overall result is standard operation in the context of resource scarcity, making it a competitive advantage. It is evident from the above that for the development and deployment of cancer molecule-specific treatments, a prime target is the Raf kinases, notably for melanoma.[53]

A medication used as a single treatment in patients suffering from metastatic melanoma that targets B-RAFV600E (PLX4032/RG7204; Plexxikon/ Roche, Berkeley, CA, USA) has lately demonstrated spectacular results having response rates of about 70-80 per cent. Another ATP-competitive B-RAF inhibitor (GSK 2118436; GlaxoSmithKline, Brentford, UK) produced identical results. These medications are well tolerated in patients with solid tumours with mutated B-RAFV600E, like thyroid carcinomas or colon malignancies, and are presently under clinical trials. The contradictory increase in activation and proliferation of the MEK/ERK pathway in cells without mutated B-RAFV600E is an intriguing and perhaps concerning problem. The drug's allosteric action encourages endogenous B-RAF to dimerize to C-RAF or A-RAF, the underlying mechanism. It is because a single active component is only required to activate the MEK/ERK pathway within the dimers; the inhibitors activate rather than disable the pathway at non-saturating concentrations, especially when the activated Ras is present, and this mechanism may be fuelling the quick formation of benign skin tumours in RAF inhibitor-treated patient. The fact that is more concerning is the melanoma cells, which develop chemoresistance through various molecular mechanisms, resulting in drug-responsive disease recurrence. Unlike imatinib resistance, which is frequently caused due to mutations in the target BCR-kinase ABL's domain. In relapsing tumours, de novo mutations in B-RAF have not been found. Instead, acquired resistance included converting to different MEK kinases (other Raf isoforms or COT/Tpl2) or activating mutations in NRAS, as well as overexpression of receptor tyrosine kinases directing other pathways.[54]

ABL-protein contribution

ABL kinases drive tumor cell dissemination by triggering actin polymerization, promoting membrane protrusions, and altering cell adherence, migration, and invasion. Crucial for breast, hepatocellular carcinoma, and melanoma cells, ABL kinases respond to proliferative factors and chemokines, facilitating invasion and motility. MDA-MB-231 breast cancer cells particularly rely on ABL2. These kinases phosphorylate invasion-related proteins and regulate gene expression, facilitating invasion and matrix breakdown. ABL2 is crucial for matrix breakdown and invasion, localized in invadopodia of breast cancer cells.[55]

In MDA-MB-231 breast cancer cells, ABL2 depletion reduces invasion and matrix breakdown. ABL2 facilitates invadopodia maturation by linking EGFR and Src kinase, promoting cortactin tyrosine phosphorylation for actin polymerization. ABL kinases target multiple actin regulatory proteins and interact with Abi1, essential for invasion and MMP-9 production. ABL2 also modulates MMP activity by interacting with MT1-MMP, facilitating invadopodia function. Melanoma cell invasion requires both ABL1 and ABL2, regulating MMP production via STAT3-dependent and independent pathways.[56]

Another potential target of ABL kinases at invadopodia is the N-WASP (WASL) actin regulatory protein, which is phosphorylated by ABL kinases and required for actin polymerization at invadopodia mediated by Arp2/3. It was recently shown that T cell ABL kinases directly substrate and bind to HEF1 (NEDD9). HEF1 facilitates the invasion of cancer cells, and ABL-mediated phosphorylation of HEF1 may increase the invasive behavior of chemokine-induced T cell migration. The ability of ABL2 knockdown to regulate the formation and function of invadopodia was shown by the reduction in cancer cell invasion and intravasation that occurred when MDA-MB-231 cells were implanted in the mammary fat pad.[57]

Recent research trends on novel drug targets and novel molecules

Raf-1 inhibitors

Using a three-kinase-coupled experiment, Glaxo Wellcome discovered a strong A class of oxindoles that contain Raf-1 inhibitors (Table 1). The potency of the inhibitor was increased by using phenol substitutes. Low micromolar quantities of several compounds were identified to block ERK1 and ERK2 activation in cells, with IC50 values in the low nanomolar range and acidic pKa values.[58]

Additionally, Merck disclosed A-Raf-1 inhibitor that poses an ATP competition and exhibits IC50 values ranging from 0.3 to 2.0 M for anchorage-independent growth and 2 nM against recombinant Raf. Together, Bayer and Onyx developed the most sophisticated inhibitors of Raf-1. It has been demonstrated that Bay439006 works well in studies on cells and animals. Cancer patients with metastatic or locally advanced disease who

participated in Phase I trials are presently being examined for Bay439006.[59]

JNK/SAPK pathway inhibitor

Small molecule drugs that target the JNK/SAPK MAPK pathway are being developed. In vitro, SP600125 inhibits JNK2 at 100 nm but not ERK, p38, or IB kinase (IKK) at micromolar doses. Although the cellular IC50 value was micromolar, in vitro research suggests that small-molecule medication development could be feasible for the JNK family.[60]

Creating inhibitors that function at various levels in these pathways and are specific to each MAPK family will vastly improve our comprehension of these signaling cascades' intricate interplay. CEP1347 (KT7515), a 17 new small-molecule JNK pathway inhibitor, has been discovered. The mixed lineage kinase (MLK) CEP1347 inhibits family; MLK1, 2, and 3 have IC50 values ranging from 23 to 51 nM. The JNK pathway's upstream activators are MLK1, 2, and 3. As a result, this is the first attempt to inhibit MEKKs upstream of JNKs to target the JNK pathway. The abundance and diversity of genes encoding MEKKs have made identifying their precise in vivo roles challenging. As a result, it's unclear which of these enzymes will be the most effective therapeutic targets. However, RAF-1 has a large amount of evidence indicating that it functions as the principal effector of Ras, the most often mutated oncogene in malignant tumours. As a result, Raf-1 has been identified as a potential therapeutic target in cancer.[61]

MEK-inhibitors

MEK1 and MEK2 inhibitors are also being developed, although at a slower pace than p38 inhibitors. The fact that none of the MEK family members have three-dimensional structures has complicated their attempts. As a result of their apparent lack of ATP competition, two of these inhibitors- PD98059 and U0126-should have distinct binding sites on MEK; structural knowledge is especially crucial for this particular family of kinase inhibitors. Details of structural knowledge of these compounds' critical mechanisms would greatly aid the drug design process. In studies of several kinase inhibitors, it was discovered that the MEK1 and MEK2 inhibitors were most precise kinase inhibitors tested, inhibiting the fewest non-target kinases out of a panel of 24 kinases. MEKs are appealing therapeutic targets due to their extremely limited substrate specificity, in which they phosphorylate and control only a few downstream MAPKs, usually one or two. MEK1 and MEK2 activity was increased in a large percentage of primary human tumour cells. As a result, MEK1 and MEK2 inhibitors are being researched as cancer treatment agents. In an in vitro screen for ERK activation inhibitors, PD98059—the first MEK1 and MEK2 inhibitor-was found. U0126, a second inhibitor discovered shortly after, was located in a cell-based search for activator protein 1 (AP-1) inhibitors triggered by phorbol ester. ATP is not competed with by either of these inhibitors. Because labelled U0126 competes with PD98059 for MEK1 binding, they appear to bind to the same sites on the protein.[62]

While it has been demonstrated that U0126 and PD98059 suppress MEK1 phosphorylation and ERK1 and ERK2 activation, these first-generation MEK inhibitors have been extensively used *in vivo* to identify ERK1 and ERK2 biological activity. Moreover, these two inhibitors have recently been demonstrated to limit ERK5 pathway activation via direct actions on MEK5. These inhibitors are frequently utilized in cell-based research at concentrations between 5 and 50 M, where effects on ERK5 can be observed. Furthermore, PD98059 inhibits cyclooxygenase 2 (COX-2), complicating the interpretation of the inhibitory effects of PD98059.PD18435 is a MEK1 and MEK2 inhibitor of the second generation with an IC50 value of less than 20 nM, improved bioavailability, and an allosteric mode of action. With an IC50 of 120 nM, PD184352 suppressed increased ERK1 and ERK2 activity in colon 26 carcinoma cells, and it also prevented the formation of tumours from these cells when administered orally to mice. Despite no inhibition of JNK/SAPK, p38, or Akt, activation of the MEK5–ERK5 pathway by epidermal growth factor (EGF) was suppressed at micromolar doses. In vivo, PD184352 appeared to increase MEK5 phosphorylation as well. PD184352 is currently being studied in phase I clinical oncology trials.[63]

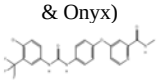
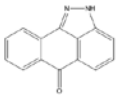
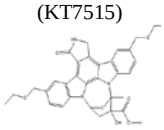
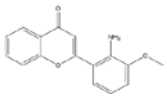
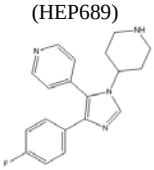
P38 inhibitor

Validation of the target and its current clinical status as evidenced by more than 48 patent filings from various companies, the most comprehensive work in MAPK inhibitor research has centered on p38. There are fifteen pharmaceutical companies. The attention to p38 stems from its significance as a signal transducer that reacts to cytokines and other cellular stressors. Several companies working on isolating kinases implicated in cell reactions to stressors such as heat shock, osmotic stress, sodium arsenite, and lipopolysaccharide (LPS) independently discovered P38. By determining the molecular target of a small-molecule inhibitor of IL-1 and TNF-α production in response to LPS, one of these research groups could extract and clone human p38.This implied that p38 is not just a potential target for therapy, but that it is also a key mediator of reactions to stimuli that cause cellular stress are expected to block not only the production but also the activities of pro-inflammatory cytokines, thus breaking the destructive cycle that is common in inflammatory and immune-responsive disorders. Hopefully, these drugs will be able to alter the molecular basis of the responses. Animal experiments and clinical trials have been used to investigate selective p38 small-molecule inhibitors. These inhibitors are effective in animal models of arthritis and other inflammatory diseases. After using these inhibitors for therapy, molecular markers of inflammatory lung disease were reduced, including eosinophil recruitment, increased cytokine output, and increased metalloproteinase activity. Several of these drugs are now being tested in clinical trials for rheumatoid arthritis. Vertex 745, which is in Phase II trials, appears to be the most advanced. SB235699 (HEP689) has also been approved for clinical trials as a topical psoriasis therapy. Those companies are proving their dedication to and faith in p38 therapies by developing second-generation backup chemicals.[64,65]

Recent computational approaches towards the least explored RAF-K pathway

The MAPK pathway, a crucial player in cellular signaling, encompasses a diverse set of serine and threonine protein kinases.

Table 1. Recent advances in drug discovery: Molecules targeting MAPK signaling pathways

Sr. No.	Molecule class	Molecule name & Structure	Function	Recent status in drug discovery pipeline	Ref
1	Raf-1 inhibitors	Oxindole derivatives (Glaxo Wellcome)	Strong inhibition of Raf-1	Discovery phase, increased potency with phenol substitutes	[66]
2	Raf-1 inhibitors	Merck inhibitor	Competes with ATP, potent inhibition	IC50 2nM against recombinant Raf, 0.3-2.0 M for anchorage-independent growth	[67]
3	Raf-1 inhibitors	Bay439006 (Bayer & Onyx) 	Effective in cell and animal tests	Phase I studies for cancer that is metastatic or locally advanced	[68]
4	JNK/SAP pathway inhibitor	SP600125 	Inhibits JNK2, not ERK, p38, or IKK	Cellular IC_{50} at micromolar doses	[69]
5	JNK/SAP pathway inhibitor	CEP1347 (KT7515) 	Inhibits MLK1, 2, and 3	First attempt to target upstream MEKKs in the JNK pathway	[70]
6	MEK inhibitors	PD98059 and U0126 	Inhibit MEK1 phosphorylation	Used for *in vivo* studies, not ATP competitors	[71]
7	MEK inhibitors	PD18435	Second-generation MEK1/2 inhibitor	IC_{50} < 20nM, improved bioavailability, phase I trials	[72]
8	P38 inhibitors	Various companies	Multiple p38 inhibitors in research	Aimed at inflammatory and immune-responsive disorders	[73]
9	P38 inhibitors	Vertex 745	Phase II trials	Effective in animal models of arthritis and inflammatory diseases	[74]
10	P38 inhibitors	SB235699 (HEP689) 	Approved for clinical trials	Used as a topical psoriasis therapy	[75]

Among these, the Raf kinases-A-, B-, and C-Raf and Raf-1-are vital components influencing cell proliferation and survival upon growth factor activation.[76,77] Their irregular transmission pathways have been implicated in various cancers like melanoma, thyroid, and ovarian, showcasing the significant role of Ras-Raf-MEK-ERK pathway mutations in cancer onset. B-Raf, a serine/threonine kinase, has garnered considerable attention due to its involvement in approximately 7% of human cancers. The prevalent V600E mutation, substituting valine with glutamic acid, notably amplifies phosphorylation 500 times compared to other B-Raf variants. Research focuses on potential inhibitors like sorafenib, PLX4720, AZ628, and SB-590885 to address this, with SB-590885 displaying a 100-fold greater efficacy in inhibiting sorafenib.[78,79] Understanding its interaction with specific amino acid residues, especially PHE583, sheds light on its inhibitory mechanism. Studies indicate the substantial anti-B-Raf kinase activity of 4, 5-dihydropyrazoles. Their potential for generating effective B-Raf kinase inhibitors, especially using the pyrazoline ring, presents a promising avenue for drug development. Pyrazoles, known for diverse industrial applications, demonstrate intriguing therapeutic potentials, spanning from muscle relaxation to anti-cancer properties.[80] Investigating novel compounds as B-Raf kinase inhibitors through drug design research uncovers essential substituents identified by a 3D QSAR study and critical features outlined in pharmacophore studies (Table 2). Computational models employing atom and field-based approaches have been pivotal in understanding B-Raf kinase interactions.[81,82] These models, delineating properties at the atomic and molecular levels, have led to identifying potent compounds against B-Raf kinase via virtual screening. Molecular docking research emphasizes the interactions of essential amino acids with ligands, utilizing advanced methodologies like MMGBSA to elucidate docking postures accurately. Exploring B-Raf kinase inhibitors through computational studies has unveiled promising drug discovery and design avenues. These endeavours, spanning from identifying potential compounds to understanding intricate molecular interactions, offer valuable insights into combating diseases associated with aberrant MAPK signaling, especially in cancer therapeutics.[83,84]

Table 2. Computational approaches and findings in exploring the B-Raf kinase pathway

Sr. No.	Computational approach	Key Findings	Relevant interaction
1	3D QSAR	Identified essential substituents on the pyrazoline ring	-
2	Pharmacophore study	Identified crucial properties for interaction with B-Raf kinase	-
3	Molecular docking	Determined essential amino acids for B-Raf kinase inhibition	Interactions with Cpds C1, C12, and C14: PHE 583, CYS 532, SER 536, and ASP 594
4	Virtual screening (VS)	Discovered potent ZINC compounds against B-Raf kinase	-
5	M-Dock (Molecular docking)	Revealed interactions of B-Raf kinase essential amino acids with ligands	Interaction details with compounds
6	Atom and field-based models	Utilized for computational studies	-

The investigation in the mentioned computational approaches uses the PHASE module to formulate 3D pharmacophore hypotheses, specifically AAHRR 1, which encapsulates pivotal properties crucial for hindering B-Raf kinase activity. Through the employment of 3D-QSAR models, crucial properties governing the inhibitory action on B-Raf kinase, encompassing atom type fractions, steric, electrostatic, HBD and HBA, and hydrophobic traits, were comprehensively elucidated, employing both atom and field-based QSAR methodologies.[85,86] The receptor-ligand complex's stability was chiefly established through hydrogen bonding and stacking interactions. This comprehensive analysis facilitated the identification of potential molecules, such as C1, C12, C14, and C16n, displaying promising scores and robust binding capabilities within the active site of the targeted B-Raf kinase of significant note were the virtually screened compounds ZINC13989686 and ZINC23908357, which emerged as particularly noteworthy candidates, holding substantial promise for future developmental strides.[87] The computational analyses conducted are poised to provide valuable insights for researchers pursuing novel anticancer therapeutics.[88-92] The delineation of potential compounds and their crucial interactions for effectively targeting B-Raf kinase signifies a notable contribution to the field, offering avenues for further exploration and development in cancer treatment strategies.[93-95]

CONCLUSION

Exploring small-molecule inhibitors targeting MAPK pathway components, particularly ERK, MEK1, and MEK2 kinases, represents a promising avenue in cancer treatment. These inhibitors have shown significant potential in animal models and are now under investigation for their therapeutic efficacy in inflammatory and cancer diseases in clinical settings. The Ras-Raf-MEK-ERK pathway, a common downstream pathway for various growth factor tyrosine kinase receptors frequently altered in human cancers, presents a clear and vital therapeutic target. While historically associated with promoting growth, this pathway potentially prevents apoptosis under certain conditions. The emergence of new medications targeting this pathway, exemplified by BAY 43-9006, developed initially as a Raf kinase inhibitor but exhibiting multiple target interactions including VEGFR-2, Flt-3, and c-Kit, showcases promising anti-proliferative and antiangiogenic properties, particularly evident in vascular tumours like renal cell malignancies. This manuscript underscores the importance of understanding the ERK signaling system in both healthy and cancerous tissues, emphasizing the potential of emerging treatments that target the ERK cascade at the Raf kinase level, thereby offering new possibilities for therapeutic interventions in cancer treatment and beyond.

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AUTHOR CONTRIBUTIONS

Tuhin Mukherjee, Satyajit Mohanty, Jasleen Kaur, Mayukh Das: carried out the literature review and prepared the original draft. Tuhin Mukherjee, Satyajit Mohanty and Krishnendu Adhikary, Prity Chatterjee: Revisions and editing. Tuhin Mukherjee and Satyajit Mohanty: conceptualized, supervised, edited and critically revised the work. Dr. Rajkumar Maiti: Review and editing.

CONFLICT OF INTEREST

The authors declare that they have no financial relationships or affiliations with any organization or institution that could have a financial conflict or financial interest in the topics or materials included in the work.

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