Published in final edited form as:

Cancer J. 2016 ; 22(3): 165–174. doi:10.1097/PPO.0000000000000187.

Therapeutic Approaches to RAS Mutation

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Abstract

The study of oncogenic RAS mutations has led to crucial discoveries regarding cancer molecular biology and behavior, and has been integral in shaping the era of targeted cancer therapy. RAS mutations are one of the most common oncogenic drivers in human cancer, and intense efforts to find a clinically effective inhibitor is ongoing. Despite these efforts, targeting RAS mutations has remained elusive, so much so that some have termed oncogenic RAS mutations as “undruggable”. In this review, we will summarize current understanding of RAS biology, explore strategies to inhibit RAS oncoproteins and its downstream effectors, and discuss recently described complexities that have shed new light on this pursuit.

Keywords

RAS; synthetic lethality; MAPK; PI3K; oncogenic; colorectal cancer

Introduction

Rat sarcoma virus (*RAS*) proto-oncogenes encode 21,000-dalton membrane bound small GTPases that include Harvey-Ras (HRAS), Kirsten-Ras (KRAS), and neuroblastoma-Ras (NRAS), collectively called RAS [1]. Oncogenic mutant RAS isoforms were first described over three decades ago when they were implicated in the development of rat sarcomas [1-5]. *RAS* gene mutations are among the most frequently mutated genes in human cancers, found in approximately 30% of all tumor types and in approximately 50% of colorectal cancer (CRC) [6, 7]. Clinically, RAS mutations have proven to be an elusive target despite the substantial amount of research investigating strategies to inhibit oncogenic *RAS* mutations. In addition, oncogenic RAS mutations cause acquired resistance to anti-epidermal growth factor receptor (EGFR) therapies such as cetuximab and panitumumab [7-11]. Given its clinical relevance as an important predictive biomarker, the American Society of Clinical Oncology (ASCO) and National Comprehensive Cancer Network (NCCN) guidelines recommend RAS testing for *KRAS* and *NRAS* mutations in exon 2 (codons 12 and 13), 3 (codons 59 and 61), and 4 (codons 117 and 146) when managing patients with metastatic colorectal cancer. Due to the elusive nature of RAS biology, the National Cancer Institute

organized a RAS initiative in 2013 aimed at developing effective therapies targeted at oncogenic RAS [12, 13].

The complexities of oncogenic RAS mutations

In a sentinel paper published in 1988, Vogelstein *et al.* first described the role of RAS activation in the development of CRC [14]. Upon DNA analysis of 80 adenomas and 92 CRC samples, the authors observed *RAS* gene mutations in 58% of adenomas larger than 1 cm and in 47% of carcinomas. *RAS* mutations appear to be an early event in CRC tumorigenesis [15]. Consistent with present day data, the majority were *KRAS* mutations, although 5 *NRAS* mutations were also found. The authors concluded that RAS is often a dominating oncogene and additional aberrations, such as loss of tumor suppressing genes, are responsible for development of CRC.

RAS mutations in human malignancies encompass a variety of tumor types. For example, *KRAS* mutations exist in greater than 90% of pancreatic cancers, 44-53% of colorectal cancers, and 17-33% of lung cancers, whereas *NRAS* and *HRAS* occur less frequently and are most often observed in melanoma, acute myeloid leukemia, and thyroid cancers (Table 1) [7, 16-19]. Once present, oncogenic *RAS* mutations act through many different pathways to elicit its tumor-promoting effects and render RAS insensitive to GTPase-activating proteins (GAPs) activity, promoting constitutive activation of RAS leading to a persistent “on” signal activating multiple downstream effector proteins [20]. Tumor cells subsequently develop an “oncogenic addiction” to *RAS* activity driving tumor cell survival, differentiation, and proliferation[21].

RAS biology: structure, function, and mutations

RAS functions as signal transducers important for regulating cell proliferation, differentiation, and survival in normal and malignant cells. RAS exists in an “on” or “off” state by cycling between GTP-bound active and GDP-bound inactive forms [22]. The RAS-mitogen activated protein kinase-ERK kinase-extracellular signal-related kinase (RAS-RAF-MEK-ERK) pathway is one of the best characterized signal transduction pathways and its aberrancies are commonly implicated in the development of multiple different cancer types including colorectal cancer (Figure 1a) [22].

In 1988, de Vos *et al.* first reported the amino acid sequence and tertiary structure of HRAS-GTP using crystallography [23]. Following this tertiary structural mapping, the description of the RAS-MAPK pathway largely unfolded throughout the 1990s. By the late 1990s, it was known that binding of the epidermal growth factor receptor (EGFR) tyrosine kinase to its ligand(s) recruits guanine nucleotide exchange factors (GEFs), such as Son-of-sevenless (SOS), to promote the formation of RAS-GTP. Crystal structure analysis of the interaction between RAS, GEFs, and GAPs show that GEFs catalyze formation of RAS-GTP, the active form, and GAPs catalyze conversion back to RAS-GDP, the inactive form [24, 25].

Through interaction with GEFs, RAS undergoes a conformational change in two flexible regions called Switch I (residues 32-38) and Switch II (residues 60-75) that favors GTP binding (Figure 2) [26]. RAS-GTP then binds to RAF serine/threonine kinases, such as B-

RAF and C-RAF, leading to RAF phosphorylation and allosteric conformational changes. In turn, RAF phosphorylates and activates MEK 1/2 dual specificity protein kinase leading to phosphorylation and activation of ERK 1/2. Activated ERK 1/2 then translocates into the cell's nucleus and activates transcription factors such as E26 transformation-specific (Ets-family) proteins. Other targets of RAS include multiple downstream effector proteins such as phosphoinositide 3-kinases (PI3K), Ral guanine nucleotide dissociation stimulator (RalGDS) family proteins and phospholipase C [22, 27].

The observation that RAS-GTP exists in 2 states heightens the complexity of our understanding of RAS activity [27-30]. Regardless of the presence or absence of oncogenic mutations, RAS-GTP may exist in either State 1, or its inactive form, and State 2, or its active form. The state of RAS-GTP largely depends on the position of the Switch I and II regions. Switch I and II consist of flexible loops within the GTP-bound RAS protein and are the main interface for effector protein recognition. The flexibility of these loops presumably allows a broad range of sequence diversity in the RAS-binding domains of different effector proteins.

Post-translational lipid modification of RAS also remains an area of interest in understanding RAS biology and inhibition (Figure 1b). It has been observed that lipid modification of the C-terminus domain of RAS is crucial for intracellular membrane anchoring and downstream effector activation [31]. While the C-terminus of RAS isoforms are variable, they all terminate with the CAAX (C: cysteine, A: aliphatic amino acids, X: any amino acids) motif sequence [32]. Multiple enzymes covalently modify RAS during transportation from the nucleus to the intracellular membrane. First, farnesyltransferase (FTase) attaches a farnesyl moiety to the cytoplasmic RAS. This is followed by proteolytic cleavage of the AAX sequence by RAS-converting enzyme-1 (RCE1) and subsequent carboxymethylation on the newly formed C-terminal Cys186 by isoprenylcysteine carboxymethyltransferase-1 (ICMT1). An additional step may occur where a palmitoyl moiety attaches to the Cys residue immediately upstream of the CAAX motif via palmitoyltransferase (PTase). Interestingly, distinct RAS isoforms may undergo different post-translational lipid modification altering RAS anchoring and its behavior. Despite much research in developing targeted therapies aimed at inhibiting these enzymes, nothing has proven to be efficacious to date (see below).

Oncogenic RAS mutations lead to a protein locked in the RAS-GTP state, allowing a constitutively active protein where GAPs are no longer able to accelerate its transition back to RAS-GDP, or “off”, state. Oncogenic mutations in the nucleotides 12, 13, and 61 of the G domain prevent GAPs interaction with RAS, allowing it to maintain a GTP-bound, active state.

Ras isoforms and post-translational behavior in cancer

A recent publication by Nussinov *et al.* demonstrated evidence that small GTPase Ras isoforms might exist in different signaling states according to splice variants and tissue type [33]. The *KRAS* gene encodes for two splice variants, K-Ras4A and K-Ras4B. While it has been observed that K-ras4B is the dominant isoform in human cancer, Tsai *et al.* recently

showed both K-Ras isoforms may be equally expressed in human CRC [34]. While sequence analysis of the catalytic domains of H-Ras, N-Ras, K-Ras4A, and K-Ras4B show they are nearly identical, their hypervariable region (HVR) of their C-terminus domain may differ substantially. This variability in the HVR may lead to distinct interactions with the intracellular membrane, altering behavior and activity of the different Ras isoforms. There is also evidence that K-Ras4A may exist in two different states, and that depending on the tumor type the oncogenic K-Ras4A may be K-Ras4B-like, in which K-Ras4A is only farnesylated, or N-Ras-like, in which K-Ras4A is palmitoylated and farnesylated. Nussinov *et al.* suggest that adenocarcinomas such as colon, pancreatic, and lung cancer may express oncogenic K-Ras4A that mirrors a K-Ras4B-like state versus tumor types such as melanoma and acute myeloid leukemia. This variation among differing K-Ras4A states may have important biological and clinical implications, as protein interactions and signaling transduction via K-Ras4A protein may differ depending on its state and tumor type. Clearly, the emerging complexities of RAS isoforms and their biology in different cancer types further complicate the ability to therapeutically target RAS.

Targeting RAS

Small molecule RAS inhibition has largely been centered around three main concepts, 1) inhibiting membrane targeting of RAS, 2) preventing the formation of RAS-GTP via blockade of RAS-GEF interaction, and 3) blocking RAS-effector protein interaction. Alternative inhibition of RAS mutations has included using combinational approaches, synthetic lethality, and immunotherapy. Below we discuss strategies for each approach.

Targeting RAS-membrane interaction

RAS activity is reliant upon correct intracellular localization to the inner leaflet of the plasma membrane. The transportation of cytoplasmic to membrane-bound RAS is regulated by post-translational lipid modifications. Targeting the enzymes responsible for these modifications was first described in the late 1990s. As previously mentioned, lipid modification occurs in the HVR of RAS. Only later the observation was made that only 15% homology existed in the HVR of different RAS isoforms, highlighting the presence of multiple different series in which RAS could undergo lipidations. To further complicate matters, certain RAS isoforms, such as K-Ras4B, undergo an additional palmitoylation step.

Multiple FTase inhibitors (FTIs) have shown promising preclinical data, some of which reached late phase clinical trials, but none have ultimately proven to be efficacious to date [35, 36]. James *et al.* showed that FTIs inhibited downstream effects of HRasV12-mutated Rat-1 cells while leaving unmutated cells unaffected [37]. Interestingly, FTI anti-tumor effects were not seen in KRasBV12-mutated Rat-1 cells, suggesting that response to FTI inhibition depends on the presence of distinct RAS isoforms [38]. In addition, preclinical data has shown that FTI effects may be circumvented via alternate prenylation by geranylgeranyl transferase I (GGTase I) [35]. Prenylation allows effective membrane localization of KRAS and NRAS in the presence of FTI. Targeting of GGTase I as a single-agent has failed to show any clinical efficacy to date [39]. Preclinical studies showed that dual inhibition of FTI and GGTase I blocks translocation of RAS, but also led to excessive

toxicity[40]. Inhibitors targeting palmitoylation of HRAS and NRAS elicited phenotypic reversion in HRASG12V-transformed fibroblast cell line; however, their anti-tumor effects in human cancer cell lines or xenografts have yet to be determined [41]. Inhibition of other enzymes responsible for lipidation, including RCE1 and ICMT, have failed to show good preclinical data compared to other FTIs [42].

Given the lack of efficacy targeting inhibitors of enzymes for farnesylation and palmitoylation, attention has been placed on inhibiting transport proteins. Salirasib, a farnesylcystein mimetic interfering with the binding of farnesylated K-Ras4B to the Ras-escort protein Galectin, has at best shown modest clinical efficacy in early phase trials both as a single-agent and in combination with conventional chemotherapy [43, 44]. In addition, RAS dislodgement from the plasma membrane and subsequent cytoplasmic degradation was not clearly shown in human trials. Zimmermann *et al.* further optimized compounds to target transport and discovered deltarasin, a molecule capable of inhibiting the interaction of K-Ras4B with the escort protein phosphodiesterase 6 delta (PDE δ) [45]. Deltarasin demonstrated both *in vitro* and *in vivo* activity through inhibition of Ras-dependent signaling, although deltarasin's effects have been observed to be nonspecific and inhibit multiple other GTPases [45, 46].

Direct RAS inhibition

The paradigm for inhibiting mutated or overexpressed proteins in “oncogenic addicted” malignant cells are attractive for two major reasons, 1) deprivation of the oncogene with a specific targeted agent leads to cell arrest and death, and 2) normal cells without the “addiction” would not be harmed by the therapy. For these reasons, much attention has been made to the development of a direct RAS inhibitor. The development of direct RAS inhibitors have been fraught with road blocks, in which it has earned its reputation as “undruggable”, given the protein's extremely high (picomolar) affinity for guanine nucleotides, the 10-fold higher cellular concentration of GTP compared with GDP, and the lack of available drug binding sites on the activated RAS-GTP protein.

The usefulness of the inhibition of RAS-GDP to RAS-GTP remains unclear given the nature of constitutively activated oncogenic RAS mutations. However, some argue that certain cancers remain dependent upon wild-type (WT) RAS expression for cell maintenance and survival [47]. Through crystallography, multiple compounds have been isolated to target nucleotide exchange between GEFs and RAS-GDP. Optimization of structure-based derivatives by Ostrem *et al.* revealed a previously unrecognized pocket adjacent to the Switch 2 region, called the SII pocket [48]. Through allosteric binding, this group of agents increases the affinity of KRASG12C for GDP over GTP [49]. However, these agents have showed nonspecific binding in follow-up studies thus far.

To bypass this issue, investigators have attempted to develop agents that target one specific oncogenic RAS mutation through covalent inhibition [48, 50]. K-RasG12C, a mutation found in approximately half of all KRAS-mutant lung cancers, has recently become an exciting target due to the amenable nature for covalent modification of its thiol group Cys12. Lim *et al.* showed that SML-10-70-1 was capable of mimicking GDP and covalently binding with KRASG12C, trapping it in an inactive form. The ability for covalent bonding allows

this compound to overcome the picomolar affinity of RAS for guanine nucleotides. SML-10-70-1, a pro-drug of SML-8-73-1, reportedly also demonstrated the ability to inhibit proliferation of KRasG12C bearing cells; however, further preclinical studies have shown that SML-10-70-1 may act nonspecifically given its anti-proliferative effects in other RAS mutation types [50].

Another agent that has shown promise is a potent acrylamide, named AA12. AA12 induced apoptosis in K-RasG12C cell lines *in vitro*, but not in cell lines with WT RAS [48]. Additional analysis has shown that AA12 may also act non-specifically. In an alternative approach, the inhibition of formation of the RAS-GTP complex has been studied using synthetic hydrogen bond surrogate (HBS) peptides. Designed by Bar-Sagi et al., HBS3 inhibited the Sos-Ras interaction and down-regulated the RAS-MAPK signaling in response to EGF stimulation [51]. Clearly, all of these agents listed above need to undergo optimization to allow suitable pharmacokinetic and pharmacodynamic properties prior to their use in the clinical setting. Despite these setbacks, there remains much excitement regarding the therapeutic potential for these classes of drugs.

Inhibition of downstream effector interactions

The most promising therapeutic strategy for targeting oncogenic RAS mutations thus far has been blockade of downstream effectors that interact with RAS due to the aforementioned difficulties of inhibiting oncogenic RAS mutations via transport or direct RAS targeting. Driven by the knowledge that RAS-GTP most prominently interacts with MAPK and phosphoinositide 3 kinase (PI3K) pathways, substantial preclinical and clinical research has been directed at the inhibition of the active components of these signal transduction pathways. Multiple agents already currently available that target different kinases in the MAPK and PI3K pathways.

With the aid of nuclear magnetic resonance (NMR) spectroscopy and crystallography, much research has been dedicated to structure analysis and investigation of possible agents that bind to the activation site of RAS. While multiple compounds have been discovered, the therapeutic effects of these compounds largely remain a mystery. For example, sulindac, a nonsteroidal anti-inflammatory drug, was reported to inhibit HRAS-induced malignant transformation of MDCK-F3 cells and Ras-dependent Raf activation; however, there has been lack of experimental evidence to show anti-tumor effects [52, 53].

As previously described, RAS-GTP exists in dynamic equilibrium between state 1 (inactive form) and state 2 (active form), and it is now known that RAS-GTP may be vulnerable to inhibitor binding while in state 1 due to the presence of an exposed pocket, which is absent in state 2. Recently, the discovery of Kobe0065-family compounds, which inhibit Ras-Raf binding, has shown inhibited Ras-Raf association in cells and decreased phosphorylation on the RAS-RAF-MEK-ERK pathway [54]. Moreover, these compounds have also reportedly inhibited Ras-PI3K-AKT and RAS-RAL-GDS-RALA/B pathways, in addition to RAS-SOS interaction. It has also been observed that these compounds inhibited the proliferation of colorectal and pancreatic cancer cell lines carrying oncogenic *Ras* mutations and showed anti-tumor effects in a xenograft model of K-RasG12V mutated human colon cancer [55].

The RAL pathway has also been implicated in a variety of cancers and aberrant activation of this pathway through oncogenic RAS is felt to lead to more aggressive tumor phenotypes [56]. Through interaction of activated RAS with RAL-GDS (RAL-guanine nucleotide dissociation stimulator), the downstream effectors Ras-like GTPases, RALA and RALB, two homologous G-proteins, are activated that then promote cell survival and proliferation. Yan *et al.* recently discovered a unique pocket in RAL-GDP susceptible to allosteric inhibition and found a group of small molecules that blocked RAL-GDP nucleotide exchange that hindered activation of RALA or RALB and showed anti-tumor effects in RAL-dependent human cancer cell lines and xenograft models [57].

Inhibiting kinases in the MAPK pathway have also been in intense focus for therapeutic targets. RAF kinases interact with RAS through a RAS binding domain, leading to activation of MEK 1/2 followed by ERK that then acts on transcription factors to promote gene expression. Drugs targeting components of the MAPK pathway include BRAF and MEK inhibitors. While single-agent MEK inhibitors have not shown promise in BRAF-mutant mCRC, as demonstrated in a phase II study by Kopetz *et al.*, preclinical and clinical activity with MEK inhibition has been observed in *RAS* mutant cancer [58]. In a phase I trial using single-agent trametinib, a MEK inhibitor, Infante *et al.* demonstrated an overall response rate of 10% in solid tumors that included KRAS-mutant pancreatic and NSCLC patients; however, none of the 13 patients with KRAS-mutant CRC showed a response [59]. In a phase II open label study, treatment with single agent MEK inhibition led to a 20% response rate in *NRAS*-mutant melanoma [60]. The combination of MEK inhibition and docetaxel in patients with KRAS mutant metastatic NSCLC has also shown benefit in a recently published phase II clinical trial [61]. Unfortunately, combination MEK plus conventional chemotherapy has not shown improved efficacy in KRAS-mutant pancreatic cancer [62].

Similarly, single-agent targeting of the PI3K pathway in RAS-driven malignancies has proven ineffective in the clinical setting. Negative feedback mechanisms, overexpression of alternate key regulator proteins, and reflexive activation of other downstream partners of RAS likely account for the bulk of this resistance [63-65]. Alternatively, preclinical data using dual inhibition of PI3K and MEK in KRAS-mutant cell lines and xenograft models have shown benefit, in addition to modest benefit observed in pancreatic cancer mouse models [64, 66]. This data has spurred ongoing preclinical and clinical research using a combination inhibitor strategy targeting kinases such as MEK, ERK, PI3K, AKT, and/or mTOR [67]. In another recently reported phase Ib trial, combination of a PI3K plus a MEK inhibitor demonstrated an overall response rate in 29% of patients with KRAS-mutated ovarian cancer [68]. This degree of response was not seen in lung or pancreatic cancer patients. The toxicity of this combination was also suspect as 65% of patients experienced a grade 3/4 adverse event, and the authors predict that the recommended phase 2 dose may require quick dose reduction in further trials. While this strategy has shown only modest clinical benefit with moderate toxicity thus far, this strategy has been adopted in many ongoing clinical trials with highly anticipated results [69, 70].

Synthetic lethality screens

Synthetic lethality occurs when combination of mutations in two or more genes lead to cell death, while a mutation in only one of these genes is viable. In oncogenic RAS mutant cancers, the targeting of a key-signaling molecule may elicit cell death, whereas normal cells would not be vulnerable. Large-scale knockdown of signaling molecules in oncogenic RAS mutant cancers have been studied [71-76]. As with other strategies, producing specific and reproducible anti-tumor effects has proven to be difficult.

Using large-scale screening of short hairpin RNA (shRNA) technology, Barbie *et al.* identified TBK1, a tyrosine kinase that acts downstream of the RAS/RALGDS/RALB axis, as a synthetically lethal partner of mutant KRAS [72, 77]. TBK1 appears to regulate growth KRAS mutant cells via the chemokine CCL5 and cytokine interleukin-6. Mometinib, a dual JAK2 and TBK1 inhibitor, combined with a MEK inhibitor showed regression in growth of KRAS-driven lung cancers in xenograft models. This combination is currently being studied in early clinical trials (NCT02258607).

Corcoran *et al.* also used large scale screening of shRNA to discover genes that acted synergistically with MEK inhibition to induce apoptosis in RAS-driven cancer cells [71]. This screen identified BCL-XL, an anti-apoptotic gene, as a potential target. It has been observed that single-agent MEK inhibition induces expression of BIM, a pro-apoptotic gene, but this isn't enough to induce apoptosis. When selumetinib was combined with anti-sense RNA to BLM-XL, KRAS mutant cell lines underwent apoptosis. This effect was reproduced when navitoclax, an inhibitor of antiapoptotic proteins BCL-2 and BCL-XL, was combined with selumetinib. A clinical trial combining navitoclax and trametinib, a MEK inhibitor approved for melanoma treatment, is currently underway (NCT02079740).

Eckhardt et al. has also shown that the Wnt pathway plays an important role in KRAS-mutant CRC [78]. Through shRNA screening, targeting the Wnt pathway increased sensitivity to selumetinib, and the combination of Wnt and MEK inhibition showed synergistic anti-tumor effects in *in vitro* and *in vivo* KRAS-mutant colorectal cancer models [78].

Another method investigating genetically engineered mice has been used to take advantage of synthetic lethality. Puyol *et al.* recently showed that interphase cyclin-dependent kinases (CDKs) were necessary for survival and proliferation in KRAS mutant NSCLC[76]. They also showed that loss of CDK4 halted cell proliferation in mutant cells and were able to recapitulate this effect by treating with a CDK4 inhibitor in a mouse model. Kwong et al. also demonstrated that combination of a cyclin-dependent kinase 4 (CDK4) inhibition with MEK inhibition led to significant effects [79]. This research has led to a clinical trial using palbociclib, a CDK4/6 inhibitor already FDA-approved for use in estrogen receptor-positive metastatic breast cancer, combined with PD-0325901, a MEK inhibitor (NCT02022982).

Lastly, there has been recent research showing that RAS-driven tumor dependence on autophagy, the digestion of intracellular components by fusion with lysosomes for nutrient support required for tumor homeostasis and growth [19, 80]. While studies using

hydroxychloroquine, an autophagy inhibitor, have largely been unimpressive, more potent anti-autophagy agents are in development [81, 82].

Immunologic approaches

In the early 1990s, it was recognized that a T cell immune response could be induced through presentation of peptides from oncogenic RAS proteins [83, 84]. It was discovered that mice vaccinated with oncogenic RAS protein with codon 12 arginine were protected from developing tumors with the same mutation, but not from tumors harboring other RAS mutations and that CD8+ T cell depletion removed this effect [84]. Vaccination of patients with pancreatic cancer *in vivo* with RAS peptides of the same oncogenic RAS mutation also demonstrated a mutant RAS-specific T cell response, measured by interferon-ELISPOT, in 2 out of the 5 patients [85]. This did not lead to any antitumor responses, however. Similar outcomes were seen in pancreatic cancer patients who received antigen-pulsed antigen-presenting cells as the immunogen [86].

In another approach, patients were administered mutant RAS peptides together with granulocyte-macrophage colony-stimulating factor as a method for stimulating T cell immune response. While immune responses (measured by ELISPOT or proliferative assay) were observed in the majority of patients, no anti-tumor effects were appreciated. Vaccination therapy has also been tested in the adjuvant setting in RAS-mutant CRC and pancreatic cancer patients, but the authors concluded that they were unable to prove that the vaccine influenced disease recurrence rates [87].

While vaccine therapy has largely been disappointing, there is much optimism in harnessing checkpoint inhibitors to treat oncogenic RAS mutant cancers. Targets such as CTLA4, PD-1, and PD-L1 have led to dramatic responses in a variety of tumor types, and the basis for these responses appear to be polyclonal recognition of proteins and not from a single peptide [88]. There has been some discussion about whether adding vaccine therapy to this approach would compound therapeutic effects [18]. It remains to be seen whether immunotherapy with or without vaccination therapy will be efficacious in oncogenic RAS-mutant cancers.

Summary

The pursuit to understand RAS biology and discover RAS oncoprotein inhibitors has proven to be extremely difficult. With information gathered from each new failed attempt at RAS inhibition, the puzzle for finding clinically effective inhibitors becomes more complex. Initially, oncogenic RAS behavior was seemingly organized in a linear, unidirectional signal transduction pathway. We now know RAS biology is much more complex, and its effects are exerted in a highly dynamic, bi-directional network of kinases with a high degree of redundancy and feedback loops enabling RAS-driven cancers to develop therapeutic resistance regardless of therapeutic strategy.

Despite many obstacles in targeting oncogenic RAS mutations, researchers and clinicians remain optimistic in inhibiting RAS through a variety of strategies. While single inhibitor therapy has largely failed in RAS-driven cancers, dual inhibition with MEK and PI3K inhibitor therapy has shown some efficacy (and toxicity) in preclinical and clinical trials (see

Table 2 for a list of selected current clinical trials). The discovery of a pocket in Switch II of the RAS-GTP protein is a promising target for potential agents to allosterically inhibit RAS. RAS trafficking protein targets such as PDE δ and Galectin have also been identified and may become a more relevant treatment option with ongoing research. Synthetic lethality has become a valuable method in both understanding RAS biology in addition to finding possible effective targets including the CKD, BCL-XL, and TBK1 families. One of the most promising approaches in drug discovery are the GDP analogue agents that covalently bind to specific RAS mutations locking RAS in an inactive form. Vaccine therapy has not demonstrated any clinical efficacy to date; however, checkpoint inhibitor therapy in combination with RAS mutant peptide vaccine therapy may offer a new approach in immunotherapy in RAS-driven cancers. Given the potential impact that RAS inhibition could have on millions of patients with cancer worldwide, it is no surprise that this is a rapidly evolving field with its own NCI-designated center to track progress (www.cancer.gov/research/key-initiatives/ras).

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Figure 1. RAS-mediated signal transduction pathways and post-translational modification of RAS. (A) The RAS-RAF-MEK-ERK is commonly implicated as the primary signaling pathway of RAS. Ligand activated RTKs in turn activate GEFs, which catalyze the formation of RAS-GDP, the inactive form, into RAS-GTP, the active form. RAS-GTP then acts on multiple downstream effectors, including PI3K and RAL pathways. (B) Post-translational modification of RAS C termini include multiple enzyme-catalyzed lipidation processes. Farnesylation (blue diamond) occurs on the Cys of the conserved CAAX region of the C termini. RAS then undergoes a proteolytic cleavage of AAX and carboxymethylation. The isoforms HRAS, NRAS, and KRas4A additionally undergo palmitoylation (orange diamond). PDE δ and Galactin escort RAS for its ultimate recruitment into the plasma membrane. * Indicates enzymes currently targeted in studies for RAS inhibition related to RAS trafficking.

Figure 2. Structure of RAS. RAS exists as a molecular switch cycling between RAS-GDP and RAS-GTP, classically thought of as “off” and “on”, respectively. Recent data has shown that RAS-GTP may exist in equilibrium between two states dictated by conformational changes in flexible loop regions named Switch 1 and Switch II. RAS-GTP State 1 is in active form without known targetable pockets, whereas State 2 is an inactive form that possesses a recently discovered pocket near Switch II that may lead RAS-GTP vulnerable to allosteric inhibition.

Table 1 Approximate frequency of *RAS* mutations in human cancers^[1, 18, 54]

Cancer Type	% <i>KRAS</i>	% <i>NRAS</i>	% <i>HRAS</i>	% All <i>RAS</i>
Pancreatic adenocarcinoma	90-98	0-0.5	0	91-98
Colorectal adenocarcinoma	40-45	4-8	0	44-53
Multiple myeloma	22	19	0	42
Lung adenocarcinoma	16-33	0.6-0.9	0.3-0.5	17-33
Skin cutaneous melanoma	0.8	28	1	29
Biliary carcinoma	25	3	0	27
Uterine endometroid carcinoma	14-21	2-3	0.4-0.5	16-25
Small intestine adenocarcinoma	23	0.7	0	23
Chronic myelomonocytic leukemia	9	13	0	22
Thyroid carcinoma	1-2	6-9	4	13-14
Acute myeloid leukemia	3-4	7-11	2	11-15
Cervical adenocarcinoma	7-8	0.8	0-6	7-15
Urothelial carcinoma	3-4	1-2	6-9	11-15
Stomach adenocarcinoma	6-11	1	0-1	9-12
Head and neck squamous cell carcinoma	0.5-2	0.3-2	5-6	5-10
Gastric carcinoma	4.0-6	1	0-1	5-9
Esophageal adenocarcinoma	2-4	0	0.6-0.7	3-5

Table 2

Selected current clinical trials using targeted agents in RAS-mutant cancers

Phase	Trial Name	NCT No.	Sponsor	Agent 1	Agent 2	Disease Setting
I	MEK162 and RAF265 in Adult Patients with Advanced Solid Tumors Harboring RAS or BRAFV600E Mutations	NCT01352273	Novartis Pharmaceuticals	MEK inhibitor MEK162	BRAF and VEGFR2 inhibitor RAF265	Advanced solid tumor harboring BRAF V600E or RAS mutations
I	A Study to Investigate Safety, Pharmacokinetics (PK) and Pharmacodynamics (PD) of BKM120 Plus GSK1120212 in Selected Advanced Solid Tumor Patients	NCT01155453	Novartis Pharmaceuticals	MEK inhibitor GSK1120212	PI3K inhibitor BKM120	Advanced NSCLC, ovarian, and pancreatic cancer with RAS or BRAF mutation
I	Safety, Pharmacokinetics and Pharmacodynamics of BEZ235 Plus MEK162 in Selected Advanced Solid Tumor Patients	NCT01337765	Novartis Pharmaceuticals	MEK inhibitor MEK162	PI3K/mTOR inhibitor BEZ235	Advanced solid tumors with KRAS, NRAS, or BRAF mutations
I	A Phase I Study of MEK 1/2 Inhibitor PD-0325901 With cMET Inhibitor PF-03241066 in RASMT and RASWT (With Aberrant c-MET) Colorectal Cancer Patients	NCT02510001	University of Oxford	MEK inhibitor PD-0325901	c-MET inhibitor PF-03241066	RAS-mutant or MET over-amplified advanced CRC
I	Safety, Pharmacokinetics and Pharmacodynamics of BKM120 plus MEK162 in Selected Advanced Solid Tumor Patients	NCT01363232	Novartis Pharmaceuticals	MEK inhibitor MEK162	PI3K inhibitor BKM120	Advanced solid tumors with KRAS, NRAS, or BRAF mutations
I	Clinical Study of PI3K/mTOR Inhibitors in Combination With an Oral MEK Inhibitor or Irinotecan in Patients with Advanced Cancer	NCT01347886	Pfizer	MEK inhibitor PD-0325901	PI3K/mTOR inhibitor PF-05212384	Once MTD is determined, further testing will be done in KRAS-mutant colorectal cancer
I	A Combination Therapy Study of MK-2206 and AZD6244 in Participants With	NCT01021748	Merck Sharp & Dohme	MEK inhibitor selumetinib	AKT inhibitor MK-2206	Advanced refractory solid malignancies; part 2, KRAS-mutant NSCLC

Phase	Trial Name	NCT No.	Sponsor	Agent 1	Agent 2	Disease Setting
	Advanced Solid Tumors (MK-2206-010)					
I/II	Sorafenib With Irinotecan in Metastatic Colorectal Cancer (mCRC) and K-RAS Mutation (NEXIRI)	NCT00989469	Institut du Cancer d Montpellier	Sorafenib	Irinotecan	KRAS-mutant mCRC subsequent-line therapy
I/II	MEK Inhibitor MSC1936369B Plus FOLFIRI in Second Line KRAS Mutated Metastatic Colorectal Cancer (mCRC)	NCT01085331	EMD Serono	MEK inhibitor MSC1936369B or placebo	FOLFIRI	KRAS-mutant mCRC second-line therapy
I/II	A Phase Ib Study of MEK162 Plus BYL719 in Adult Patients With Selected Advanced Solid Tumors	NCT01449058	Novartis Pharmaceuticals	MEK inhibitor MEK162	PI3K inhibitor BYL719	Advanced cancer with RAS or BRAF mutation
I/II	Phase I/II Study of Tipifarnib [ZARNESTRATM, Farnesyltransferase Inhibitor R115777 (NSC702818)] in Patients With Myeloproliferative Disorders	NCT02210858	National Cancer Institute	Farnesyltransferase inhibitor Tipifarnib	N/A	Patients with myeloproliferative disorders
I/II	An Open Label, Two-Part, Phase Ib/II Study to Investigate the Safety, Pharmacokinetics, Pharmacodynamics, and Clinical Activity of the MEK Inhibitor Trametinib and the BCL2-Family Inhibitor Navitoclax (ABT-263) in Combination in Subjects With KRAS Mutation-Positive Advanced Solid Tumors	NCT02079740	National Cancer Institute	MEK inhibitor Trametinib	BCL2 inhibitor Navitoclax	KRAS-mutant advanced tumors
II	Refametinib (BAY86-9766) in RAS Mutant Hepatocellular Carcinoma (HCC)	NCT01915589	Bayer	MEK inhibitor refametinib	N/A	RAS-mutated, advanced HCC
II	Pilot Study of the Combination of MK-2206, an AKT inhibitor, and AZD6244,	NCT01333475	National Cancer Institute	MEK inhibitor selumetinib	AKT inhibitor MK-2206	KRAS WT versus MT advanced colorectal cancer

Phase	Trial Name	NCT No.	Sponsor	Agent 1	Agent 2	Disease Setting
	a MEK inhibitor, in Patients With Advanced Colorectal Carcinoma					
II	A Multicenter, Open Label, Randomized Phase II Trial of the MEK Inhibitor Pimasertib or Dacarbazine in Previously Untreated Subjects With N-RAS Mutated Locally Advanced or Metastatic Malignant Cutaneous Melanoma	NCT01693068	EMD Serono	MEK inhibitor pimasertib	Dacarbazine	NRAS-mutant locally advanced or metastatic cutaneous melanoma
II	An Open Label Phase II study of Tipifarnib in Patients With Previously-Treated, Advanced, HRAS mutant Urothelial Carcinoma	NCT02535650	Samsung Medical Center	Farnesyltransferase inhibitor Tipifarnib	N/A	HRAS-mutant urothelial carcinoma in subsequent-line setting
II	Trametinib and AKT Inhibitor GSK2141795 in Treating Patients with Acute Myeloid Leukemia	NCT01907815	National Cancer Institute	MEK inhibitor trametinib	AKT inhibitor GSK2141795	AML with RAS mutations
II	A Dose Finding and Phase II Study of Selumetinib (AZD6244) in Combination With Irinotecan in 2 nd Line Patients with K-ras or B-raf Mutation Positive Advanced or Metastatic Colorectal Cancer	NCT01116271	AstraZeneca	MEK inhibitor selumetinib	Irinotecan	KRAS- or BRAF-mutated advanced or mCRC in the second-line setting
II	A Phase II Study of Sorafenib in Combination With Cetuximab (Erbix) in EGFR Expressing Metastatic Colorectal Cancer (CRC)	NCT00326495	National Cancer Institute	VEGFR2 inhibitor Sorafenib	Cetuximab	KRAS-mutant advanced CRC in the subsequent line setting
II	Single-arm Study of Selumetinib in Combination With Docetaxel, in Advanced Gastric Adenocarcinoma Patients With Low/High MEK Signature, RAS	NCT02448290	Samsung Medical Center	MEK inhibitor selumetinib	Docetaxel	Advanced, RAS mutant or RAS amplified gastric adenocarcinoma in the second-line setting

Phase	Trial Name	NCT No.	Sponsor	Agent 1	Agent 2	Disease Setting
	Mutation or RAS Amplification as a Second-line Chemotherapy					
II	A Phase II Study of Single Agent MEK162 in Patients with Advanced Melanoma	NCT01320085	Novartis Pharmaceuticals	MEK inhibitor MEK162	N/A	NRAS- and BRAF-mutant advanced melanoma. Overall response rate of 20% in both NRAS- and BRAF-mutant disease (see reference 55)
II	A Pilot Phase 2 Trial of Immunogenicity, and Safety of GI-4000; an Inactivated Recombinant S. Cerevisiae Expressing Mutant Ras Protein, as Consolidation Therapy Following Curative Treatment for Stage I-III Non-Small Cell Lung Cancer (NSCLC) With Tumor Sequence Confirmation of K-ras Mutation	NCT00655161	GlobeImmune	Vaccine therapy GI-4000	N/A	Stage I-III KRAS-mutant NSCLC status-post curative treatment
II	Safety and Efficacy Study of BMS-908662 Alone or in Combination With Cetuximab in Subjects With K-RAS or B-RAF Mutation Positive Advanced or Metastatic Colorectal Cancer	NCT01086267	Bristol-Myers Squibb	RAF inhibitor BMS-908662	Cetuximab	KRAS- or BRAF-mutated advanced or metastatic CRC
III	Study Comparing the Efficacy of MEK162 Versus Dacarbazine in Unresectable or Metastatic NRAS Mutation-positive Melanoma	NCT01763164	Novartis Pharmaceuticals	MEK inhibitor MEK162	Dacarbazine	Unresectable or metastatic NRAS Q61-mutant melanoma.
III	Assess Efficacy & Safety of Selumetinib in Combination With Docetaxel in Patients Receiving 2 nd Line Treatment for v-Ki-ras2 Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) Positive NSCLC (SELECT-1)	NCT01933932	AstraZeneca	MEK inhibitor selumetinib or placebo	Docetaxel	KRAS-mutant advanced NSCLC, second-line therapy

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Abbreviations: CRC, colorectal cancer; AML, acute myeloid leukemia; FOLFIRI, fluorouracil, leucovorin, and irinotecan; MTD, maximum-tolerated dose; mTOR, mammalian target of rapamycin; N/A, not applicable; NSCLC, non-small-cell lung cancer; ORR, overall response rate; PI3K, phosphoinositide 3 kinase; VEGFR2, vascular endothelial growth factor receptor 2.