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Pharmacological Targeting of RAS: Recent Success with Direct Inhibitors.

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Abstract

RAS has long been viewed as undruggable due to its lack of deep pockets for binding of small molecule inhibitors. However, recent successes in the development of direct RAS inhibitors suggest that the goal of pharmacological inhibition of RAS in patients may soon be realized. This review will discuss the role of RAS in cancer, the approaches used to develop direct RAS inhibitors, and highlight recent successes in the development of novel RAS inhibitory compounds that target different aspects of RAS biochemistry. In particular, this review will discuss the different properties of RAS that have been targeted by various inhibitors including membrane localization, the different activation states of RAS, effector binding, and nucleotide exchange. In addition, this review will highlight the recent success with mutation-specific inhibitors that exploit the unique biochemistry of the RAS(G12C) mutant. Although this mutation in KRAS accounts for 11% of all *KRAS* mutations in cancer, it is the most prominent KRAS mutant in lung cancer suggesting that G12C-specific inhibitors may provide a new approach for treating the subset of lung cancer patients harboring this mutant allele. Finally, this review will discuss the involvement of dimerization in RAS function and highlight new approaches to inhibit RAS by specifically interfering with RAS:RAS interaction.

Keywords

RAS inhibitor; cancer; effector interaction; nucleotide exchange; GTPase; monobody; CAAX motif

1. Introduction

The RAS family of genes (*HRAS*, *KRAS* and *NRAS*) is a frequent target of mutational insult in cancer. Nearly 30% of human tumors possess mutations in *RAS* with some cancers such as pancreatic cancer having *RAS* mutations in nearly 100% of tumors. *RAS* genes encode a 21 kDa protein possessing GTPase activity. Normally, RAS proteins reside in an inactive, GDP-bound state on the plasma membrane in quiescent cells. However, following

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mitogenic stimulation, guanine nucleotide exchange factors (GEFs), such as SOS, are recruited to RAS resulting in release of GDP and formation of a transient nucleotide-free state (Fig. 1A). Given the higher cellular concentrations of GTP vs GDP, RAS proteins subsequently load with GTP. This nucleotide exchange results in significant conformational changes in two specific regions of RAS referred to as Switch 1 (SW1; amino acids 30–40) and Switch 2 (SW2; amino acids 60–76) (Fig. 1B). When bound to GTP, these regions engage specific RAS effector proteins resulting in the subsequent activation of these RAS targets. Signaling from RAS is terminated through hydrolysis of GTP, which is mediated by the intrinsic enzymatic activity of RAS. However, RAS is a relatively poor enzyme and is aided in this process through the action of GTPase activating/accelerating proteins (GAPs) that enhance the intrinsic enzymatic activity of RAS by nearly 100-fold thereby returning RAS to its inactive GDP-bound state.

Oncogenic activation of RAS occurs predominantly through missense mutations in codons 12, 13, or 61. These changes result in a shift of the protein to the active GTP-bound state resulting in constitutive engagement and activation of RAS effector pathways. These mutant RAS proteins are not only important for driving tumor formation but also for maintenance of the transformed phenotype both *in vitro* in tumor cell models [1–4] and *in vivo* mouse models [5–9]. Thus, RAS has long been a central target for therapeutic inhibition. Despite significant efforts over several decades, there remains a lack of FDA-approved anti-RAS therapeutics. However, recent findings provide renewed hope that RAS inhibitors will eventually be deployed in the clinic.

1. RAS structure

The three *RAS* genes encode 4 highly homologous proteins (HRAS, KRAS4A, KRAS4B and NRAS) (Fig. 1B). The first 172–174 amino acids of the 4 proteins constitute the G-domain, which is nearly identical between the proteins, with only a few differences. This region can be divided into two distinct regions: an effector lobe (amino acids 1–86) which is identical among the RAS isoforms, and an allosteric lobe (amino acids 87–172) which diverges slightly (86% identity). The COOH-terminal hypervariable regions (HVRs) are the most divergent regions of RAS isoforms. The HVR is essential to RAS function, targeting RAS to membranes as a result of posttranslational lipidation of the COOH-terminal Cys of the CAAX motif (Cys, aliphatic, aliphatic, any amino acid). Farnesylated RAS is initially targeted to the endoplasmic reticulum (ER) where RAS is further modified by RAS converting enzyme (RCE1), which proteolytically removes the three COOH amino acids, followed by methylation of the carboxyl group of the terminal Cys residue by isoprenylcysteine carboxymethyltransferase (ICMT) [10]. HRAS, NRAS, and KRAS4A are then differentially modified by additional lipidation steps. KRAS4A and NRAS possess an additional Cys residue (C180 and C181, respectively) that is modified by the attachment of a single palmitoyl group whereas HRAS has two additional Cys residues (C181 and C184) that are targeted for palmitoylation. KRAS4B lacks these Cys residues and instead possesses a stretch of basic amino acids that serves as a second membrane targeting signal to facilitate association of farnesylated KRAS4B with the plasma membrane. These COOH-terminal modifications and association with the plasma membrane are essential for RAS function as

mutation of the Cys of the CAAX motif abolishes RAS signaling and transforming activity [11].

Although RAS proteins share significant sequence identity, it is clear that RAS isoforms are not equivalent [12–14]. While mutant forms of each *RAS* gene are highly transforming in experimental systems, *KRAS* is the most frequently mutated isoform in human cancer: 83% of all *RAS* mutations are in *KRAS*, 13% in *NRAS*, and 4% in *HRAS* (Fig. 1C) [15]. Furthermore, each *RAS* gene exhibits a different pattern of mutation in cancer. Codon 12 mutations are most frequent in *KRAS* whereas codon 61 mutations are more frequent in *NRAS*. In the case of *HRAS*, mutations in codons 12, 13, and 61 occur with roughly equal frequency (Fig. 2)[15]. The frequency of specific mutations also varies significantly between *RAS* genes (Fig. 2B). G12D mutations are most prominent in *KRAS* where as Q61R and G13R mutations are the most frequent in *NRAS* and *HRAS*, respectively. Furthermore, the spectrum of mutations in a *RAS* isoform differs between cancer types. For example, *KRAS*(G12D) mutations predominate in pancreatic cancers whereas lung cancers more frequently are characterized by *KRAS*(G12C) mutations [16]. These observations suggest that *KRAS* mutation provides a greater selective advantage to tumor cells *in vivo*.

Despite the identity of SW1 and SW2 effector regions, RAS isoforms differentially engage specific effectors. For example, *KRAS* activates RAF proteins (BRAF and CRAF) better than *HRAS* whereas *HRAS* preferentially activates the PI3K pathway [17, 18]. These differences may stem in part from the divergent HVRs that target each RAS isoform to specific lipid raft regions of the plasma membrane. Such partitioning may limit the interaction of RAS to specific effectors at each site [19–22]. The HVRs also appear to influence effector engagement [23, 24]. Given the preponderance of *KRAS* mutations in cancer, most attention in recent years has centered on targeting this specific isoform.

2. Targeting RAS membrane localization

Structural studies of RAS indicated a lack of deep pockets for binding of small inhibitory molecules. This observation led to the initial belief that RAS may be refractory to direct inhibition [25]. Given the necessity of RAS association with the plasma membrane for its oncogenic activity [11], initial attempts to develop pharmacological inhibitors of RAS focused on inhibiting the enzyme responsible for the posttranslational modification of the CAAX motif, namely farnesyltransferase (FTase)[26]. Initial preclinical studies with FTase inhibitors (FTIs) demonstrated success in blocking tumor cells both *in vitro* and *in vivo* [27, 28]. However, these results did not depend on RAS mutations. Furthermore, membrane localization of both *KRAS* and *NRAS* was unaffected by FTIs [29]. These results foreshadowed the disappointing clinical trial with FTIs (see refs in [30]).

The lack of efficacy of FTIs was due in part to the surprising biochemistry underlying the posttranslational modification of the CAAX motif. While *HRAS* was dependent on the COOH-terminal lipidation mediated by FTase, FTI treatment of *KRAS* or *NRAS* resulted in alternative lipidation of these proteins by geranylgeranyltransferases (GGTases) thus bypassing the FTase [29, 31, 32]. As a result, FTIs have proven ineffective in solid tumors, which predominantly harbor *KRAS* mutations. Although FTIs have been combined with

geranylgeranyltransferase inhibitors (GGTIs), this approach suffers from dose limiting toxicities likely due to finding that >100 proteins serve as prenylation targets of these enzymes [33]. Thus, these inhibitors will likely suffer from prohibitive off-target effects thereby limiting their clinical effectiveness. Despite the lack of success thus far in clinical trials, FTIs are being evaluated for use in specific malignancies with *HRAS* mutations, *e.g.* bladder cancer, renal cell cancer, and thyroid cancers. Given the dependence of HRAS on FTase, there remains optimism for potential clinical success of FTIs on a more narrow selection of malignancies. However, concerns remain regarding toxicity due to the inhibition of the myriad additional substrates of FTase.

Although FTIs have been unsuccessful in blocking KRAS localization, a recent study provided some support to the possibility of specifically inhibiting KRAS lipidation with modified FTIs [34]. Incorporation of an electrophilic moiety into the FTI resulted in specific reaction with the CAAX motif of KRAS but not HRAS thereby blocking farnesylation and geranylgeranylation of KRAS resulting in moderate mislocalization of KRAS to the cytosol. Although the effects on KRAS-mediated activation of ERK and proliferation were minimal, these promising results suggest that further modifications and refinements of this approach may result in a more potent inhibitor of KRAS lipidation and function.

Despite their lack of success in cancer, FTIs have shown some efficacy in treatment of other diseases. Hutchinson-Gilford Progeria Syndrome (HGPS) is a rare genetic disease characterized by premature aging, short stature, cardiac defects, low body weight, early hair loss, and early death [35]. HGPS arises from a defect in lamin A in which a COOH-terminal Cys remains persistently farnesylated rendering the protein non-functional [36, 37]. This discovery prompted the testing of FTIs as a potential therapeutic approach. Treatment of mouse models of HGPS improved the phenotype of affected animals [38]. Clinical trials using lonafarnib demonstrated an improvement in weight gain or other secondary outcomes (*e.g.*, decreased arterial pulse wave velocity, increase in skeletal rigidity) [39] and improvement in lifespan, although many of the phenotypes associated with HGPS remained [40]. Thus, the possibility remains that FTIs may receive eventual FDA approval in limited clinical settings.

An alternative approach has emerged for interfering with the plasma membrane localization of RAS proteins, particularly KRAS. The delta subunit of phosphodiesterase 6 (PDE δ) binds the farnesylated tail of KRAS and functions in part as a chaperone to facilitate proper KRAS membrane localization [41, 42]. An inhibitor of PDE δ (Deltarasin) disrupted KRAS: PDE δ binding resulting in mislocalization of KRAS and reduced signaling in *KRAS* mutant cancer cells [43]. Second generation PDE δ inhibitors have been isolated with lower toxicity and greater selectivity toward inhibiting KRAS mutant cancer lines [44, 45]. However, a number of issues remain with these compounds. PDE δ regulates not only KRAS but also HRAS and NRAS [41]. In addition, it is unclear how many additional farnesylated proteins interact with PDE δ raising similar concerns as seen with FTIs regarding the ability of PDE δ inhibitors to selectively target RAS.

3. Targeting different activation states of RAS

RAS proteins cycle between three states: an inactive GDP-bound state, a transient nucleotide-free state, and an activate GTP-bound state that engages effector proteins. NMR studies revealed that RAS adopts distinct conformational states upon GTP loading [46]. In State 1, the SW1 region adopts an open conformation that favors nucleotide exchange and inhibits effector binding. In State 2, SW1 adopts a closed conformation over the nucleotide that promotes effector binding and GTP hydrolysis. When RAS adopts the State 2 conformation, e.g., upon binding RAF, the SW2 region adopts two distinct states: T state and R state. In the T state, RAS is catalytically incompetent whereas in the R state RAS is catalytically active. Inhibitors have been identified that target different activation states of the protein. One of the first of these activation state inhibitors was Zn-cyclen which binds State 1 of RAS thereby lowering the affinity of RAS for RAF [47, 48]. However, Zn-cyclen is not a drug-like compound and thus not a viable RAS inhibitor for *in vivo* use.

Recent work identified a previously unrecognized pocket in the SW2 region of RAS, referred to as SII-P, present in the GDP-bound, inactive form. Using a novel tethering approach that selectively targeted the Cys mutation at codon 12, thiol-reactive compounds were identified that covalently bound RAS(G12C) versus wild type or other mutant RAS proteins [49]. Biochemical analysis revealed that these compounds targeted SII-P in the GDP-bound form of the protein, disrupting both SW1 and SW2, thereby preventing SOS-mediated GTP-loading and engagement of effectors. These results revealed that oncogenic RAS proteins are not locked in the GTP-bound state but rather continue to cycle through the GDP-bound state, albeit at significantly reduced rates compared to the wild type protein. Indeed, RAS(G12C) retained significant intrinsic GTPase activity compared to other oncogenic RAS mutants but remained insensitive to GAP-stimulated hydrolysis [50]. Although the pharmacological properties of the initial SII-P G12C inhibitory compounds resulted in limited activity in cells, subsequent derivatives were isolated with improved cellular activity [51, 52]. The most recent iteration of the SII-P G12C inhibitor, ARS-1620, exhibited the necessary pharmacokinetic and pharmacodynamic properties to drive KRAS(G12C) into a nearly complete GDP-bound state resulting in selective inhibition of KRAS(G12C) mutant cell lines and tumor models [53]. These results provide a significant step forward in the development of a direct RAS(G12C) inhibitor for eventual introduction into the clinic.

The SII-P inhibitors revealed that RAS(G12C) proteins do not remain locked in the GTP-loaded state in perpetuity. However, it is becoming clear that a number of additional oncogenic RAS proteins continue to cycle between the GTP- and GDP-bound states [54]. These “cycling mutants”, e.g., G13D, Q61L, are characterized by high spontaneous exchange activity, resulting in the release of GTP from the protein. Although these mutants re-load with GTP due to its higher cellular concentration vs GDP, this property has several potential consequences to RAS function. First, the rapid release of nucleotide may reduce the amount of time that these cycling mutants are bound to GTP and able to interact with effectors, thereby dampening their signaling activity. In addition, the rapid cycling properties may render such mutants independent of the action of exchange factors to stimulate reloading of RAS. As such, these cycling mutants may be refractory to inhibitors targeting

nucleotide exchange (see below). However, rapid cycling may render such mutants more amenable to inhibition with compounds that trap the nucleotide-free state. Indeed, biochemical studies examining the action of the cell-permeable compound BIM-46187 on heterotrimeric G-protein activity revealed that this compound inhibited $G\alpha_q$ by trapping the GTPase in a nucleotide-free conformation [55]. Design of molecules that target RAS when nucleotide-free may provide a viable approach to inhibit these rapid cycling mutants.

4. Targeting RAS effector binding and nucleotide exchange

A number of approaches have been utilized to identify direct RAS inhibitors that either block effector interaction or nucleotide exchange. Sulindac, an NSAID, was one of the first compounds shown to inhibit HRAS [56]. Subsequent studies demonstrated that its metabolite, sulindac sulfide, directly inhibited RAS activation of RAF and reduced RAS-mediated transformation *in vitro* [57]. Derivatives of sulindac, such as IND12, exhibited lower cyclooxygenase-inhibitory activity but enhanced RAS inhibitory activity [58]. However, relatively high concentrations (10–50 μM) were required for their biological effects indicating a lack of potency for these compounds.

MCP1 was identified by yeast two-hybrid screening for compounds that blocked RAS:RAF interaction [59]. MCP1, or derivatives of this compound, inhibited RAS:RAF interaction, RAS activation of RAF, and RAS-mediated transformation [60–62]. However, these compounds also lacked potency and will require further improvements to generate a viable RAS inhibitor.

Computational analyses of RAS structures have identified potential ligand binding pockets and lead compounds targeting such pockets [63, 64]. Shima and colleagues used an *in silico* screening approach to identify Kobe0065 [65]. This compound, and the related Kobe2602, bound State 1 of RAS and blocked RAS:RAF interaction as well as SOS-mediated nucleotide exchange. Another computational docking screen identified the pan-RAS inhibitor 3144 that targeted three sites in RAS around SW1 and the nucleotide binding pocket, disrupting RAS effector interactions and reducing RAS-driven tumors in genetically engineered mouse models [66]. Thus, these computational drug discovery approaches have identified several promising lead RAS inhibitory compounds for further development.

RAS inhibitors have also been developed that target nucleotide exchange. HBS3, a synthetic α -helix, targeted the SOS-interacting region of RAS. Interestingly, this compound preferentially bound nucleotide-free HRAS ($K_d=28 \mu\text{M}$) better than HRAS-GDP ($K_d=158 \mu\text{M}$), reducing nucleotide exchange *in vitro* and modestly reducing ERK activation in cells. Fragment-based screening approaches led to isolation of compounds targeting the same hydrophobic pocket of KRAS as recognized by the Kobe compounds [67, 68]. One of these compounds, DCAI, inhibited nucleotide release as well as nucleotide exchange on KRAS. Treatment of cells with DCAI reduced RAF RBD recruitment to the plasma membrane and decreased EGF-stimulated RAS activation [67]. Jansen and colleagues identified KRAS inhibitory compounds that bound to the same pocket as DCAI [69]. One of these compounds, cmpd2, inhibited KRAS signaling with moderate affinity. Interestingly, cmpd2 exhibited low binding affinity to KRAS in solution (690 μM) but significantly higher affinity

(0.8 μ M) to prenylated RAS immobilized on liposomes [69]. Cmpd2 bound both membrane and KRAS to promote an orientation of KRAS that occluded the effector binding region, thereby inhibiting KRAS interaction with effector targets [70].

A recent study aimed at identifying compounds targeting the SII-P site in both RAS-GDP and RAS-GTP resulted in isolation of lead compounds targeting an extended groove near SII-P, referred to as SII-G [71]. These compounds were identified using a similar tethering approach that led to identification of G12C covalent inhibitors [49]; however, in this case a modified RAS protein, [HRAS(M72C)], was utilized to identify compounds targeting the distal end of SII-P in non-G12C mutant forms of RAS. The lead compound, 2C07, covalently bound both GDP and GTP states of RAS(M72C) resulting in conformational distortions in SW2. These changes disrupted SOS binding and exchange on RAS-GDP but did not affect RAS-GTP binding to the RAF-RBD which is mediated predominantly by SW1 interactions. In contrast, a derivative of 2C07 inhibited RAS-GTP activation PI3K *in vitro*, likely due to the more extensive interaction of PI3K with both SW1 and SW2 [72]. Interestingly, the authors also identified a compound that reversibly competes with 2C07 for binding to RAS(M72C) but not wild type suggesting that this molecule may serve as a lead compound for the identification of HRAS specific inhibitors targeting this SII-G region [71].

One of the first identified RAS inhibitors was the RAS neutralizing monoclonal antibody Y13–259 that blocked effector binding [73–76]. Despite the inhibitory activity of this antibody, its inability to penetrate cells precluded use as a therapeutic. However, a cell-penetrating antibody was recently developed that inhibited signaling and oncogenic transformation by RAS both in cells and animals models [77]. This success was built upon the isolation of single domain variable fragments of antibodies, intrabodies, that specifically bound and inhibited RAS-mediated signaling and oncogenic transformation [78–80]. Crystallographic analysis revealed that this RAS inhibitory intrabody bound SW1 and SW2 domains to block effector interactions [79]. This intrabody was subsequently used in a screen to identify lead RAS inhibitory compounds that targeted this same region. A series of drug-like compounds were identified that bound the same region identified in previous screens for RAS inhibitors [67, 68]. Several of these lead drug-like compounds inhibited oncogenic RAS interaction with various effector proteins and blocked RAS-dependent signaling and transformation [81, 82]. However, further studies will be needed to optimize the pharmacodynamic properties of these compounds for potential clinical use.

5. RAS dimerization- an Achilles' heel?

A new dimension to RAS function has emerged as a potential therapeutic target to inhibit RAS in cancer cells, namely dimerization. RAS proteins are commonly referred to as monomeric GTPases given their lack of associated subunits as seen with the heterotrimeric class of G-proteins that mediate the action of G-protein coupled receptors. Indeed, solution structure and biophysical studies indicated that RAS, or at least the G-domain of RAS, existed as a monomer in solution [83–86]. In addition, structural studies suggested a biochemical mechanism for RAS action that did not require dimerization of the GTPase [25, 87–90]. The discovery that activated RAS, *i.e.*, RAS-GTP, bound the NH₂-terminus of RAF provided a direct link between RAS activation and activation of RAF [75, 91–94]. RAS was

proposed to function simply to recruit RAF to the plasma membrane where it became activated and stimulated MEK and subsequent ERK activation [95]. However, the details of the precise mechanism by which RAS activated RAF at the membrane remained undefined.

Although RAS binding to RAF was required for RAF activation, RAS binding alone was insufficient to stimulate RAF activation *in vitro* [91]. Furthermore, RAS promoted RAF activation in cells through formation of hetero- and homo-dimeric RAF complexes [96, 97]. Consistent with these findings, structural and biochemical experiments demonstrated that catalytic activation of RAF required dimerization through a side-to-side dimeric interface [98]. Each RAF monomer contains a RAS-binding domain that is capable of binding to one RAS molecule. These findings raised the question of how monomeric RAS promoted RAF dimerization and activation.

Various findings over the years have suggested that RAS may function as a dimer, or high order multimer, in the activation of its effectors. RAS dimers were initially proposed by Santos and colleagues using radiation target analysis [99]. Further work by Chataway and Baritt using purified, iodinated RAS revealed that monomeric RAS existed in equilibrium with a small amount of a polymeric species [100]. A chemoenzymatically modified, farnesylated KRAS lipoprotein formed dimers in solution [101] although a more recent study demonstrated that fully farnesylated and methylated KRAS4B purified as a monomer in solution [102] and remained monomeric even when immobilized onto membranes at different densities and lipid compositions in the absence of any effector molecules [103]. Nevertheless, a number of studies suggested involvement of RAS dimerization in the activation of RAS effectors. Artificial dimerization of RAS resulted in RAF activation in a cell-free system whereas monomeric RAS did not [104]. In addition, RAS proteins segregated into distinct nanoclusters at the plasma membrane that were important for regulating spatiotemporal signaling from RAS [105]. RAS dimers have also been observed in many X-ray crystal structures [86, 106, 107]. Our recent analyses of these structures revealed the presence of a specific RAS dimer mediated by the $\alpha 4$ and $\alpha 5$ helices of RAS in “active state” structures [107]. Recent superresolution microscopy studies provided further evidence for the formation of RAS dimers at the plasma membrane leading to RAF activation [108, 109]. However, the COOH-terminal tail, in the absence of the G-domain, was sufficient to promote dimerization of a heterologous fluorescent protein raising the question of the importance of the RAS G-domain in mediating dimerization.

While these studies suggested that RAS may indeed dimerize, the lack of an available experimental tool to block dimerization has limited study of this mechanism. However, we have developed a powerful reagent for interrogating this potential dimerization mechanism for RAS function. Using monobody technology [110], we sought to identify functionally critical regulatory sites in RAS that could be targeted for inhibition. We recently reported the isolation of a monobody, NS1, that specifically targeted the $\alpha 4$ - $\beta 6$ - $\alpha 5$ region of RAS, hereafter referred to as the $\alpha 4$ - $\alpha 5$ interface [107]. The NS1 binding interface overlapped with a proposed dimerization interface identified in RAS crystal structures [86, 106, 107]. Expression of NS1 in cells disrupted RAS dimerization and nanoclustering and inhibited oncogenic RAS activation of both RAF-MAPK and PI3K-AKT signaling pathways [107]. In the case of RAF, this inhibition was due to the inability of RAS to induce RAF

heterodimerization and activation in the presence of NS1. The importance of the $\alpha 4$ - $\alpha 5$ interface for RAS biological activity was further highlighted by the ability of NS1 to inhibit oncogenic RAS-mediated transformation both *in vitro* and *in vivo* ([107] and I. Khan and J.P. O'Bryan, unpublished observations). These findings provided the first direct evidence for the critical importance of the $\alpha 4$ - $\alpha 5$ interface in RAS self-association and function and suggested that targeting this region may provide a new approach to inhibit RAS function [111].

A recent study [112] aimed at understanding the tumor suppressor activity of wild type KRAS [113] provided additional evidence in support of RAS dimerization through this $\alpha 4$ - $\alpha 5$ interface. Using mutations predicted to disrupt RAS dimerization (D154Q), the authors demonstrated that dimerization-impaired forms of wild type KRAS no longer suppressed tumorigenesis by mutant KRAS. Introduction of these same mutations into oncogenic KRAS reduced the ability of cells expressing only these double mutant KRAS alleles to form tumors in athymic nude mice. Interestingly, the dimerization of wild type KRAS with the oncogenic KRAS reduced the sensitivity of lung cancer cells to MEK inhibitors. Elimination of wild type KRAS or expression of a dimerization-impaired allele of wild type KRAS increased the sensitivity of cells to MEK inhibitors [112]. These findings further support the importance of the $\alpha 4$ - $\alpha 5$ interface of RAS for its biological function.

Despite these findings, there remains considerable debate regarding the ability of RAS to dimerize through the G-domain. Tethering of RAS (aa 1–166) to itself using ethyleneglycol linkers of varying lengths to create dimers of the protein failed to uncover significant interactions between the G-domains [86]. It should be noted that in these studies, only GDP-loaded RAS was examined for dimerization. Given the presence of the $\alpha 4$ - $\alpha 5$ dimer only in active state RAS structures [107], it would be interesting to examine whether addition of GTP to these artificially linked RAS proteins results in detectable self-association of the G-domains. However, a recent study found that natively expressed and fully processed GTP-loaded K-RAS4B bound to supported lipid bilayer membranes remained monomeric over a range of densities and membrane compositions [103]. These findings suggest that RAS does not possess intrinsic dimerization potential. However, the authors leave open the possibility that in cells, RAS dimerization may be facilitated by additional cellular factors.

So how can we reconcile these apparent conflicting findings on RAS dimerization? One potential explanation may be that the G-domain of RAS does not physically interact as predicted by the RAS crystal structures (Fig. 4B). Rather, the G-domains may move in sufficient proximity to promote RAF heterodimerization. This association may be facilitated by RAS nanoclustering mediated by the COOH-terminal tails [114, 115]. Such a model would explain the lack of self-association observed with the RAS G-domain. In addition, this model is consistent with the inhibitory activity of NS1 monobody which may create sufficient steric hindrance to prevent two RAS protomers from moving in sufficient proximity to facilitate RAF heterodimerization and activation. Nevertheless, it appears clear that formation of higher order RAS complexes is necessary for activation (and dimerization) of RAS effectors in cells. Whether this is due to intrinsic dimerization of RAS or dimerization mediated by additional cellular co-factors remains to be determined.

6. A promising future for anti-RAS therapies

Although the disappointing clinical trial results with FTIs cast a dark shadow on anti-RAS therapies, the resurgence in identification of direct RAS inhibitors has led to very encouraging results. The most promising thus far appear to be the SII-P inhibitors targeting KRAS(G12C) mutants [53]. Although this mutation represents only 11% of all KRAS mutations in cancer, it is the most prominent KRAS mutation in lung cancer (~36% of KRAS mutations in lung; see cancer.sanger.ac.uk) [15]. Thus, such G12C-specific inhibitors may provide a new approach to treating lung cancer patients harboring this allele. Furthermore, the emergence of additional RAS inhibitory compounds, a number of which exhibit efficacy in model cell systems, provides significant hope for the eventual development of a therapeutic for what was once viewed as an “undruggable” target. While there are many reasons to remain optimistic regarding development of *bona fide* RAS inhibitors, RAS-dependent cancers are likely to acquire resistance to such treatments as observed in many cancers treated with targeted therapeutics [116]. Thus, it will be important to continue to develop new and more effective inhibitors of the critical signaling pathways that drive cancer cells. Nevertheless, the future looks promising for the eventual development of direct RAS inhibitors that can be deployed in the clinic in the battle against cancer.

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Non-standard abbreviations

SOS	Son-of-Sevenless
PI3K	phosphatidylinositol 3'-kinase
MAPK	mitogen activated protein kinase
HVR	hypervariable region
GEF	guanine nucleotide exchange factor
GAP	GTPase activating or accelerating protein
FTase	farnesyltransferase
FTI	farnesyltransferase inhibitor
GGTase	geranylgeranyltransferase
GGTI	geranylgeranyltransferase inhibitor
HGPS	Hutchinson-Gilford Progeria Syndrome
SW1	switch 1

SW2	switch 2
RBD	RAS binding domain
RCE1	RAS converting enzyme 1
ICMT	isoprenylcysteine carboxymethyltransferase
PDEδ	delta subunit of phosphodiesterase 6

7. References

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Chemical compounds studied in this article

[Deltarasin](#), PubChem CID 73292904

[Zn-cyclen](#), PubChem CID 129651749

[ARS-1620](#), PubChem CID 132274053

[BIM-46187](#), PubChem CID 11593027

[Sulindac sulfide](#), PubChem CID 5352624

[IND12](#), PubChem CID 76715657

[Kobe2601](#), PubChem CID 163309612

[3144](#), PubChem CID 102004330

[DCAI](#), PubChem CID 1381961

[ABD7](#), PubChem CID 134812710

Figure 1. RAS Proteins.

A) GTPase cycle. Normally, RAS proteins reside in the GDP-bound or inactive state. Following mitogenic stimulation by growth factors, GEFs are recruited to the plasma membrane. Bind of GEFs to RAS results in destabilization in nucleotide binding leading to the release of GDP from RAS and creation of a transient nucleotide free state. Given the high concentration of GTP in cells relative to GDP, RAS proteins load with GTP resulting in the switch to the active state. RAS-GTP recruits and activates it downstream targets such as RAF and PI3K. Termination of RAS signaling occurs through hydrolysis of GTP to GDP. Although RAS possesses intrinsic GTPase activity, it is a poor enzyme. This inactivation step is aided by GTPase accelerating/activating proteins which enhance the GTPase activity of RAS by nearly 100-fold, returning RAS to the inactive, GDP-bound state. **B)** RAS family members. KRAS4A and KRAS4B are derived from alternative splicing of the same gene resulting in different C-termini. Grey shading highlights residues that are identical in all four RAS proteins. SW1, switch 1 region (aa 30–40); SW2, switch 2 region (aa 60–76); HVR, hypervariable region. Proteins were aligned with Clustal multiple alignment. **C)** Mutation frequency in RAS alleles. Data were compiled from the Catalogue of Somatic Mutations (COSMIC), v86 [15].

Figure 2. Distribution of RAS mutations in human tumors.

A) Frequency of mutations in codons 12,13, and 61 in each RAS allele. **B)** Distribution of specific codon mutations in each RAS isoform. Data were compiled from the Catalogue of Somatic Mutations (COSMIC), v86 [15].

Figure 3. RAS-inhibitor structures.

Structures of RAS in complex with various inhibitors. Molecular surface of RAS is shaded according to different functional regions: light grey, effector lobe; dark grey, allosteric lobe; SW1, light blue; SW2, dark blue. Ligands are represented as stick models colored yellow. Nucleotide is represented as stick models in tan. The orientations of RAS differ in sets of panels to highlight the binding surfaces of each ligand: Panels **A** and **B** are in same orientation; Panels **C**, **D**, and **E** are in same orientation; and Panel **F** is in a different orientation from all other panels. Additionally, HRAS is shown at a smaller scale in **F** than depicted in panels **A-E** to highlight the binding surface of NS1 relative to the total protein. **A**) KRAS(G12C):ARS1650 (PDB: 5V9U). **B**) HRAS(M72C):2C07 (PDB: 5VBE). **C**) HRAS(T35S):Kobe2601 (PDB: 2LWI). **D**) KRAS(G12D):DCAI (PDB: 4DST). **E**) KRAS(Q61H):ABD7 (PDB: 6FA4). **F**) HRAS:NS1 (5E95).

Figure 4. Proposed model for RAS activation by self-association.

Following mitogen stimulation or oncogenic mutation, RAS becomes loaded with GTP leading to the recruitment of RAF monomers. Two RAS protomers either **A)** physically interact through specific amino acid side chains or **B)** move in close enough proximity to facilitate dimerization of downstream targets such as RAF. Binding of NS1 monobody (not shown) in either circumstance would provide significant steric hinderance to prevent RAS-mediated dimerization of RAF, thereby inhibiting RAF activation. Although not depicted in these models, it is possible that RAF is recruited to RAS only after RAS dimerizes, as previously suggested [108].