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Direct Inhibition of RAS: Quest for the Holy Grail?

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Abstract

RAS GTPases (H-, K-, and N-RAS) are the most frequently mutated oncoprotein family in human cancer. However, the relatively smooth surface architecture of RAS and its picomolar affinity for nucleotide have given rise to the assumption that RAS is an “undruggable” target. Recent advancements in drug screening, molecular modeling, and a greater understanding of RAS function have led to a resurgence in efforts to pharmacologically target this challenging foe. This review focuses on the state of the art of RAS inhibition, the approaches taken to achieve this goal, and the challenges of translating these discoveries into viable therapeutics.

Keywords

RAS inhibitor; RAS biologics; RAS monoclonal antibody; high-throughput screening; cancer

1. Introduction

RAS oncogenes were first identified as the transforming genes of the Harvey and Kirsten strains of oncogenic retroviruses,^{1,2} which transduced the cellular genes H-RAS and K-RAS, respectively. A third RAS gene, N-RAS, was subsequently identified in neuroblastoma tumors.^{3,4} Together, these three oncoproteins, collectively referred to as RAS, represent the most commonly mutated oncoproteins in human cancer. Nearly 30% of human tumors possess activating mutations in RAS with some cancers such as pancreatic ductal adenocarcinomas having K-RAS mutations in nearly 100% of tumors.⁵

RAS functions as a membrane anchored molecular switch, cycling between the inactive GDP-bound state and the active GTP-bound state (Fig. 1). Activation of RAS occurs through the recruitment of guanine nucleotide exchange factors (GEFs) to the plasma membrane (PM). For example, growth factor binding and subsequent activation of receptor tyrosine

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kinases (RTKs), such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), stimulate the recruitment of GEFs to RAS, which promotes the release of nucleotide and subsequent binding to GTP due to the 10-fold higher cellular level of GTP vs GDP.^{6,7} RAS-GTP then recruits a plethora of effectors, including RAF, PI3K, RAIN, AFAD, RASSF1, TIAM1, RALGEF, PLC ϵ , RIN1, RGS12, IMPA1, to regulate diverse biological processes such as proliferation, survival, endocytosis, migration, and cytoskeletal dynamics (Fig. 1A).⁸ Signal termination occurs through the interaction of RAS-GTP with GTPase activating/accelerating proteins (GAPs) that enhance the relatively low intrinsic GTPase activity of RAS switching RAS to the GDP-bound “off” state. Thus, mutations in RAS that impair the GAP-mediated GTPase activity or hyperactivation of upstream activators of RAS contribute to inappropriate signaling by RAS.

Over the past 3 decades, numerous attempts have been made to target RAS function directly with small molecules and peptides, yet there remain no FDA-approved RAS inhibitors in the clinic. This failure has led to the perception of RAS as “undruggable”.⁵ Recent advances in drug discovery techniques, high throughput screening methods and molecular modeling have led to a plethora of small molecules, peptides and biologics that target various aspects of RAS function. These advances will likely pave the way for successful development of anti-RAS therapeutics – hopefully in the not so distant future.

2. RAS structure and function

The three RAS genes, *H-RAS*, *K-RAS* and *-RAS*, encode four RAS isoforms: H-RAS, N-RAS and two K-RAS proteins, K-RAS4A and K-RAS4B,⁵ derived from alternative exon splicing. K-RAS4B is the predominant isoform and will be referred to simply as K-RAS from here on. Each RAS protein is ~21 KDa and divided into three functional domains: the effector lobe (residues 1–86), the allosteric lobe (residues 87–166) and the hypervariable region (HVR; residues 167–188/9) (Figs. 1B & C). The effector lobe is sequentially identical between isoforms and contains the Switch 1 (SW1; residues 30–40) and Switch 2 (SW2; residues 60–76) regions. These regions change conformation upon GTP loading to allow recruitment and activation of its various effectors.^{9,10} The allosteric lobe diverges between isoforms (86% identity) and has been implicated in RAS dimerization and membrane interactions.^{11–15} Combined, the effector lobe and the allosteric lobe constitute the G-domain (residues 1–166). Recent structural studies, however, indicate that α 5 helix extends beyond amino acid 166 to residues 172–174^{16–19} suggesting that the G-domain is slightly larger than previously described. The HVR is highly divergent between isoforms and implicated in membrane targeting and orientation of the G-domain with respect to the membrane. The HVRs possess a C-terminal CAAX motif (Cys, aliphatic, aliphatic, any residue) that is post-translationally modified by the covalent attachment of a farnesyl group to the ys of the CAAX (Figs. 1B & 2).²⁰ The farnesylated tail targets RAS to the endoplasmic reticulum (ER) and is essential for RAS biological activity (Fig. 2).²¹ However, the farnesyl group alone is not sufficient for effective plasma membrane targeting.^{22,23} H-RAS has two additional sites that are palmitoylated (Cys 181 and 184) whereas -RAS and K-RAS4A each have a single palmitoylation site (Cys 181 and Cys 180, respectively) (Fig. 1B).²⁰ In contrast, the K-RAS HVR lacks lipidation sites beyond the CAAX motif and

instead possesses an uninterrupted stretch of Lys residues (Fig. 1B) that serve as a second membrane-targeting signal.²²

3. Role of RAS in cancer

The role of RAS in cancer has been well established. Around a quarter of all human cancers contain activating mutations in one of the three RAS isoforms, making RAS the most mutated oncogene family in cancer.⁵ These mutations increase the pool of GTP-loaded RAS by impairing its intrinsic GTPase activity or interfering with the GAP-stimulated GTP hydrolysis, both of which result in increased activation of pro-oncogenic signaling pathways (Fig. 3). RAS has eleven known effectors with RAF and PI3K the most studied given their involvement in tumorigenesis along with RAS (Fig. 1A).²⁴ Additionally, TIAM1, PLC ϵ and RAL-GDS play key roles in RAS-driven oncogenesis as validated in mouse and cell culture models.^{25–27}

Although all RAS isoforms interact with common effectors and share highly conserved protein sequences, it is clear that their roles in cancer are not identical.^{28,29} Initial work on RAS focused heavily on the H-RAS isoform, which in retrospect is the least mutated isoform in human cancer (~4% of RAS-mutant cancers)(Fig. 3B).^{5,30} K-RAS mutations, in contrast, account for nearly 85% of all RAS mutations in human tumors, with nearly 100% of pancreatic ductal adenocarcinoma possessing mutant K-RAS alleles.^{5,24,30,31} N-RAS is found mutated in ~11% of cancers including melanoma and multiple myeloma.⁵ Indeed, in almost all cases the overwhelming majority of RAS mutations in a given cancer are specific for one of the three major isoforms.^{5,30}

4. Direct inhibition of RAS function – a proof of principle

The discovery of monoclonal antibody Y13–259 provided the first approach to directly inhibit RAS.³² Microinjection of Y13–259 into NIH-3T3 cells blocked oncogenic H-RAS-driven proliferation and oncogenic transformation through binding to SW2 and inhibition of RAF interaction.^{33,34} Expression of an scFv fragment of Y13–259 induced aggregation and mislocalization H-RAS in cells, likely due to the low solubility of the scFv fragment in cells.³⁵ Although the reducing potential of cells and the inability of antibodies or their fragments to efficiently cross the PM render antibodies as unlikely therapeutic options for intracellular targets, recent advances have demonstrated that monoclonal antibodies can indeed be delivered into the cell and block RAS function.³⁶ These studies demonstrate the potential of isolating reagents that directly inhibit RAS function in cells.

Another monoclonal antibody developed against a synthetic peptide corresponding to viral K-RAS(G12S) (anti-p21Ser) blocked RAS function and reversed oncogenic transformation by binding to the nucleotide-free state of RAS, preventing nucleotide loading.^{37,38} Thus, despite having picomolar affinity for nucleotide, these findings suggest that nucleotide-free RAS may represent a potential pharmacological target. Indeed, this idea is supported by recent work demonstrating a potential role for nucleotide-free RAS in cell signaling.³⁹ Although RAS-GTP binds and activates Class 1 PI3Ks, H-RAS was shown to interact with a Class 2 PI3K isoform, PI3KC2 β , but only when H-RAS was nucleotide free. This interaction

resulted in mutual inhibition of both RAS and PI3KC2 β .³⁹ A number of RAS mutants, including G13D and Q61L, have high rates of intrinsic nucleotide exchange⁴⁰ raising the possibility that these mutants are not constitutively GTP-bound but rather transit through a nucleotide-free state. Thus, it may be possible to target the nucleotide-free form of RAS in cells as a means of inhibiting RAS function. However, targeting nucleotide-free RAS may lead to an unwanted side effect of activating targets such as PI3KC2 β due to loss of negative regulation by RAS.

5. Indirect inhibitors - mislocalizing RAS

Despite the promising results with monoclonal antibody inhibition of RAS in cells, structural analysis of H-RAS revealed a lack of deep hydrophobic pockets to facilitate binding of small molecule inhibitors suggesting that RAS might be “undruggable”.⁴¹ Thus, a number of groups have approached the goal of inhibiting RAS by targeting the pathways that mediate the plasma membrane localization of RAS, which is essential for its biological activity (Fig. 4).

5.1. Prenylation inhibitors.

Farnesyl transferase is the enzyme responsible for transferring a farnesyl group to the C-terminal Cys of RAS. Lipidation at this CAAX motif is essential for membrane localization and function of all RAS isoforms.^{23,42} Farnesyltransferase inhibitors (FTIs) competitively block substrate binding to farnesyltransferase (FTase) thereby inhibiting modification of its targets (Figs. 2 & 4).⁴² Unfortunately, this approach has proven ineffective in the majority of cancers due to the ability of certain RAS isoforms to undergo alternative lipidation upon FTI treatment. K-RAS, which is the most frequently mutated S isoform in human tumors (accounts for 85% of total RAS mutations), and N-RAS are alternatively modified by geranylgeranyltransferase 1 (GGTase1) upon FTI treatment.⁴³ Thus, tumors with either K-RAS or N-RAS mutations are insensitive to FTIs.⁴⁴ These results have spawned the generation of GGTase inhibitors (GTIs),⁴³ which are currently being assessed for safety and efficacy (reviewed here⁴⁵)

However, progress has been made on specifically blocking K-RAS membrane targeting. Shokat and colleagues generated modified FTIs that incorporated an electrophilic moiety capable of postranslationally modifying the CAAX motif of K-RAS in cells.⁴⁶ This modified FTI prevented the FTase from farnesylating K-RAS, but also covalently modified the CAAX of K-RAS thereby preventing geranylgeranylation. Indeed, treatment of cells with this neosubstrate, termed compound 6*, mislocalized activated K-RAS to the cytosol presumably by preventing both farnesylation and geranylgeranylation. Despite the mislocalization of K-RAS, there was minimal reduction in ERK activity. However, treatment of cells with compound 6* and Lovastatin, which reduces concentrations of farnesyl pyrophosphate, synergistically enhanced K-RAS mislocalization, and reduced ERK activation and proliferation.⁴⁶ One continuing concern with these modified FTIs is that they may affect many targets of the FTase, including other RAS superfamily GTPases. It was noted, however, that compound 6* did not label H-RAS and may therefore be more specific to K-RAS than previous generations of FTIs.

5.2. PDE δ inhibitors.

Each RAS isoform has several membrane-targeting signals, including the C-terminal CAAX motif as well as secondary membrane targeting signals upstream of the CAAX site. However, the comparatively vast surface area of the endomembranes requires additional mechanisms to maintain a sufficient pool of RAS localized at the PM. For H-RAS and N-RAS, PM enrichment is achieved by de-palmitoylation at all membrane sites which sends H-RAS and N-RAS to the Golgi, where they are re-palmitoylated and subsequently trafficked to the PM.^{47,48} K-RAS, which is not palmitoylated, is re-localized by the delta subunit of phosphodiesterase 6 (PDE δ), which binds and solubilizes the methylated, farnesylated, C-terminal Cys of the K-RAS HVR (Fig. 2).^{49,50} K-RAS is subsequently unloaded by Arl-2 at perinuclear membranes, allowing for its re-localization to the PM.⁵⁰ In this way PDE δ actively sequesters K-RAS from endomembranes. It is important to note, however, that PDE δ also solubilizes H-RAS and N-RAS isoforms.⁴⁹ Indeed, H-, N- and K-RAS were mislocalized in liver carcinoma cells lacking PDE δ , but proper localization was rescued by ectopic PDE δ expression.⁴⁹ However, the relative importance of PDE δ versus the palmitoylation cycle for H-RAS and N-RAS localization has not been determined.

Based on this regulation of K-RAS by PDE δ , Waldmann, Bastiaens, Wittinghofer and colleagues identified a small molecule inhibitor of PDE δ , termed Deltarasin, that bound with nanomolar affinity to the farnesyl binding pocket of PDE δ and prevented the PDE δ :K-RAS association (Figs. 2 & 4).⁵¹ Deltarasin resulted in K-RAS accumulation on endomembranes, resulting in reduced proliferation and oncogenic signaling in PDAC (pancreatic ductal adenocarcinoma) cell lines.⁵¹ While Deltarasin was cytotoxic at effective doses (>5 μ M), likely due to non-specific binding to intracellular targets, a second generation PDE δ inhibitor, termed Deltazinone 1, exhibited lower cytotoxicity and greater selectivity at inhibiting K-RAS driven tumor growth.⁵² Although Deltazinone1 was active at low micromolar concentrations, its *in vitro* affinity for PDE δ was in the low nanomolar range. This discrepancy apparently arises through Arl-2 ejecting both K-RAS and Deltazinone 1 from PDE δ .⁵³ Through modification of Deltazinone 1, two derivatives were generated, Deltasonamide 1 and 2, which made additional hydrogen bonds with the farnesyl binding pocket of PDE δ , further stabilized the interaction and reduced ejection by Arl-2. Despite these changes and the picomolar affinity of Deltasonamides for PDE δ , the concentration of these compounds required to reduce proliferation in K-RAS mutant cancer lines was only slightly lower than Deltazinone 1.⁵³ However, these compounds suffered from a low partitioning coefficient that reduced their availability in the cytosol indicating that additional issues remain to be addressed for development of these compounds into effective anti-K-RAS therapeutics.

6. Direct inhibition of RAS – new hope emerges

Advances in drug design along with increased understanding of RAS structure and function has led to an overwhelming resurgence in the search for effective anti-RAS therapeutics. Indeed, The National Cancer Institute's RAS Initiative was established in 2013 with this very goal.⁵⁴ Approaches to inhibit RAS directly with small molecules have focused on

enhancing the GTPase activity of RAS, inhibiting nucleotide exchange and blocking RAS interaction with effectors (Fig. 4).

6.1. Enhancing GTP hydrolysis.

Oncogenic mutations in RAS render it predominantly GTP-bound due to loss of interaction with GAPs thereby locking RAS in the active GTP-bound state (FIG. 3A). Thus, restoration of GTP hydrolysis, either by enhancing the intrinsic GTPase activity of RAS or by restoring the sensitivity of oncogenic RAS to GAPs, is one approach to inhibit oncogenic RAS. The GTP-analog, DABP-GTP, increased the *in vitro* GTP hydrolysis of RAS mutants harboring activating mutations at codons 12 or 61 greater than the wild type (WT) form suggesting that mutant RAS was in principle capable of hydrolyzing GTP. Thus, cell permeable versions of DABP-GTP could potentially be used to enhance the GTPase activity of mutant RAS.⁵⁵ However, progress in this area has been limited.

6.2. Targeting nucleotide exchange on RAS.

Given that oncogenic mutations impair RAS GTPase activity, it may seem somewhat counterintuitive to target nucleotide exchange since mutant RAS may no longer require nucleotide cycling. However, it has become clear more recently that oncogenic RAS undergoes nucleotide cycling. Indeed, some mutants, such as K-RAS(G12C), possess significant GTPase activity and transit through a GDP-loaded state, which can be targeted by small molecule inhibitors.^{56,57}

Bar-Sagi and colleagues created a cell permeable synthetic α -helix, named HBS3, that incorporated residues 929–944 of the RAS-interacting α -helix of the RAS-GEF, SOS1 (Fig. 4).⁵⁸ HBS3 bound to a shallow cleft in the SOS-binding region of RAS, recognizing the nucleotide-free state of H-RAS with K_d of $28 \pm 4.8 \mu\text{M}$ and GDP-loaded H-RAS with K_d of $158 \pm 16 \mu\text{M}$. As a result, HBS3 blocked SOS:H-RAS interaction and reduced nucleotide exchange *in vitro*. HBS3 was cell permeable and modestly reduced levels of GTP-loaded RAS and ERK activity.⁵⁸

Walenski and colleagues isolated a cell permeable stapled peptide also based on the RAS-interacting α -helix of SOS1 (Fig. 4).⁵⁹ This peptide, termed SAH-SOS1, exhibited nanomolar affinity for K-RAS ($\sim 100 \text{ nM}$), although micromolar concentrations were required for strong reduction of EGF-driven ERK and AKT phosphorylation in K-RAS mutant PDAC cells.⁵⁹ SAH-SOS1 reduced SOS interaction with RAS and GTP binding by both WT and K-RAS(G12D).⁵⁹ Although the ability of SAH-SOS1 to reduce viability in K-RAS(G12D) mutant PDAC cells was encouraging, the *in vivo* stability of these peptide remains to be determined, along with their efficacy at inhibiting tumorigenesis *in vivo*.

Fragment-based screening led to isolation of small molecules that bound to a previously uncharacterized hydrophobic pocket on K-RAS between SW1 and SW2.^{19,60} Of note, a compound referred to as DCAI inhibited nucleotide release from K-RAS with an IC_{50} of $\sim 155 \mu\text{M}$ and prevented nucleotide exchange at an IC_{50} of $\sim 342 \mu\text{M}$, likely by interfering with RAS:SOS interaction (Figs. 4 and 5A). Further, DCAI treatment of cells reduced recruitment of the RAS binding domain-Cyc-rich domain of RAF (RAF RBD-CRD) to the PM, which corresponded to a reduction in EGF-driven RAS-GTP levels in HEK-293 cells.¹⁹

Although the affinity and potency of these compounds is low, they nevertheless reveal a potential site on RAS to target with future generations of inhibitors.

Recently, Sakamoto and colleagues isolated short inhibitory peptides: KRpep2 and KRpep2d, that had nanomolar affinity toward K-RAS(G12D) and exhibited selectivity for this mutation over WT and K-RAS(G12C).⁶¹ Although the binding interface was not determined, these peptides resulted in SOS release from K-RAS and inhibition of GTP loading suggesting that KRpep2 and KRpep2d share common interaction residues with SOS. Although extensive analysis of specificity and potency in cell was not performed, treatment of K-RAS(G12D) mutant 427 cells, but not K-RAS(G12) mutant A549 cells, with KRpep2d (30 μ) reduced ERK activation and proliferation suggesting a preference for inhibiting K-RAS(G12D). This conclusion is consistent with the observed 10-fold lower IC₅₀ value for inhibition of GTP association with K-RAS(G12D) vs K-RAS(G12). However, the inhibitory effects of these peptides were modest highlighting the need to enhance the potency and specificity of such peptides.

6.3. Targeting RAS:effector interaction.

Another approach to inhibit RAS is through blocking interaction with its effectors such as RAF and PI3K, which drive pro-proliferative and pro-survival pathways necessary for oncogenesis.²⁴ The viability of this approach was first demonstrated by the inhibitory activity of the RAS monoclonal Y13–259 as discussed earlier.^{33,34} However, subsequent work has resulted in small molecules that inhibit RAS interaction with effectors. Pei and colleagues isolated a cyclic peptide that blocked the interaction of K-RAS with the RBDs of RAF, TIAM1 and RAL-GDS *in vitro* (Fig. 4).⁶² However this compound lacked cellular activity due to poor membrane permeability. A modified version, termed Cyclorasin 9A5, potently blocked RAS:RAF association with an IC₅₀ in the low micromolar range and reduced ERK and AKT activity in RAS-mutant cancer cells (FIG. 4). Cyclorasin 9A5 reduced proliferation and induced apoptosis in H1299 lung cancer cells which possess an -AS(Q61K) mutant allele.⁶³ HSQC NMR suggested that Cyclorasin 9A5 bound the same region of RAS as DCAI.^{19,63} Cyclorasin 9A5 also blocked wild type RAS in tumor cells lines expressing a mutant EGFR. Thus, the lack of specificity for oncogenic RAS raises questions of toxicity if such a compound was used *in vivo*.

Computational screening for compounds that block the H-RAS:RAF interaction resulted in isolation of two related compounds, Kobe0065 and Kobe2602 (Fig. 4 & 5B).⁶⁴ Interestingly, these compounds bound the same hydrophobic region as DCAI¹⁹ and Cyclorasin 9A5,⁶⁵ but made somewhat different contacts with RAS (Fig. 5B). It should be noted that these results were derived from NMR analysis of a similar compound, Kobe2601, which was more water-soluble compared to Kobe0065 and 2602. These Kobe family of compounds prevented the interaction of H-RAS with RAF and blocked SOS-driven GTP loading, essentially combining the effects of the previous reagents.^{19,60,62,65} The IC₅₀ for blocking H-RAS:RAF association in H-RAS-(G12V) was roughly 10 μ M and doses of 20 μ M effectively blocked RAS:RAF interaction, ERK and AKT activity in H-RAS(G12V) transformed NIH/3T3 cells. Both Kobe0065 and 2602 compounds reduced anchorage-independent growth to varying degrees in a panel of H-, N- and K- RAS mutant cancer lines while showing significantly

less reduction in the BRAF(V600E) mutant melanoma line, A375. Finally, both Kobe compounds reduced tumor growth of the K-RAS(G12V) mutant colorectal line SW-480 in xenografts.⁶⁴

The RAS:effector interaction was targeted by the small molecule pan-RAS inhibitor, 3144.⁶⁶ This molecule was identified using computational docking methods designed to identify molecules targeting three sites around the SW1 region and nucleotide binding pocket. 3144 bound with low micromolar affinity to all three RAS isoforms, but not other RAS-related GTPases, with the exception of RRAS2. Treatment of cells with 3144 reduced RAS effector interaction. MEFs expressing only K-RAS were slightly more sensitive to 3144 (IC₅₀=3.8 μM) than RASless MEFs driven by activated BRAF (BRAF-CAAX) (IC₅₀=11 μM) suggesting potential off-target effects of this compound. Further, 3144 reduced xenograft tumor growth using metastatic breast cancer line MDA-MB-231.⁶⁶ However, this cell line harbors activating mutations in both K-RAS and B-RAF,⁶⁷ making interpretation of these result challenging. Nevertheless, treatment of KPC mutant mice with 3144 reduced tumor burden and activation of ERK and AKT suggesting that this compound possesses the necessary inhibitory activity toward oncogenic RAS to warrant further development.

6.4. RAS inhibition by guanine-nucleotide competitive inhibitors

Targeting kinases with ATP-competitive nucleotide analogs has proven highly successful over recent years.⁶⁸ Much of this success is due to the affinity of kinases for ATP being in the micromolar range. In contrast, RAS proteins have picomolar affinity for guanine nucleotides.^{69–71} This property, combined with the millimolar concentrations of GTP in cells,⁷ has led to the belief that designing GTP/GDP competitive RAS inhibitors was unlikely to be successful.⁵ However, guanine nucleotide analogs capable of competing with GTP and inhibiting RAS activity has been attempted, although these analogs showed only weak inhibition of function.^{72,73} A series of compounds (SCH-53239 and SCH-54292) were designed to compete with nucleotide.^{74,75} However, structural studies indicated that they instead bound to a cleft near SW2 of RAS with modest affinity and not the nucleotide-binding pocket. These compounds blocked nucleotide exchange, and in one case, RAS-driven proliferation. Unfortunately, their lack of potency and cytotoxic hydroxylamine group precludes their use as therapeutics, at least in their current form.

6.4. Mutation specific inhibition of RAS.

Given the importance of RAS signaling in cells, it is likely that a pan-RAS inhibitor would be quite toxic to wild type cells and therefore isolating mutation-specific RAS inhibitors would be most advantageous.^{8,76} Recently, the guanine nucleotide binding pocket of RAS has been successfully targeted with inhibitors specific to the G12C mutation. Gray and colleagues developed a thiol reactive guanine nucleotide analogue to specifically target K-RAS(G12C). This compound, SML-8–73-1, competed with millimolar concentrations of GTP, by attaching covalently to Cys12 and inserting into the nucleotide-binding pocket of K-RAS(G12C).^{77,78} However, a lack of cell permeability and inherent structural instability led to development of a second generation of these GDP mimetics that exhibited increased stability, but somewhat reduced affinity for RAS.⁷⁹ Although these compounds did not readily enter the cell, a partially “caged” derivative, SML-10–70-1, was able to enter cells

and partially inhibited ERK and AKT activation. This compound did not exhibit selectivity in blocking K-RAS(G12C) vs K-RAS(G12S) cells raising the question of specificity and potential off-target effects.⁷⁸ In addition, a recent kinetic analysis suggests that the slow covalent reaction times and loss of reversible affinity for RAS would preclude the use of these nucleotide analogues as viable therapeutic options.⁸⁰

The most promising results in targeting specific oncogenic RAS mutants has come from the work of Kevan Shokat and colleagues (Fig. 4).⁵⁷ Using a disulphide-fragment-based screening strategy, they isolated a compound (Compound 12) that did not compete with guanine nucleotides for binding to K-RAS, but instead occupied a previously uncharacterized binding pocket beneath SW2, which lies adjacent to the nucleotide binding pocket (Fig. 5C).⁵⁷ Insertion of Compound 12 into this pocket, which they named S-IIP, shifts the preference of nucleotide binding from GTP to GDP.⁵⁷ As with SML-8-73-1, Compound 12 is specific for the G12C mutation, and thus does not inhibit wild type or other oncogenic permutations of RAS. Surprisingly, Compound 12 exhibited strong preference for GDP-loaded RAS, suggesting that K-RAS(G12C) transits through a GDP-loaded state. A similar compound, ARS853, improved upon the results seen with Compound 12.^{56,81} ARS853 displayed enhanced potency toward K-RAS(G12C) mutant tumor lines compared to Compound 12.⁸¹ ARS853 was also specific for the GDP-loaded state of K-RAS(G12C), further demonstrating that at least in the case of G12C, oncogenic RAS is not constitutively GTP-loaded. Although oncogenic mutations in RAS are thought to be locked in the GTP-bound state due to impaired GTPase activity, K-RAS(G12C) retains significant intrinsic GTPase activity.^{56,82} ARS853 labeling of K-RAS(G12C) and inhibition of KRAS function required several hours of treatment suggesting that the GDP-loaded state is infrequent and that additional signals regulate K-RAS(G12C) loading with nucleotide. Indeed, activation of this mutant depends on upstream inputs from receptor activation of GEFs.⁵⁶ Thus, concurrent treatment of cells with ARS-853 and EGFR inhibitors enhanced inhibition of G12C mutant cancer cells.^{56,81} These compounds provide proof-of-principle for development of mutant-specific K-RAS inhibitors, at least against G12C mutations. Although these compounds rely on the Cys provided by this RAS mutant, the G12C mutation is present in around 12% of all K-RAS mutant cancers (Fig. 3B) and is particularly enriched in lung adenocarcinoma (44% of K-RAS mutations),⁵ making it one of the more common RAS driver mutations. Thus, development of these compounds targeting K-RAS(G12C) may have significant clinical benefit in tumors harboring this common RAS driver mutation.

7. Novel approaches to target RAS - use of biologics.

Microinjection of Y13-259 monoclonal antibody into cells provided the first demonstration that biologics might be useful in inhibiting oncogenic RAS.^{33,34} Unfortunately these reagents are not viable therapeutics due to their inability to penetrate the plasma membrane and their sensitivity to the reducing environment of cells. However, following on this initial success, single domain variable fragments of antibodies were generated that specifically bound activated RAS.⁸³ A specific clone, termed iDab#6, recognized the GTP-bound form of RAS with high affinity ($K_d=6$ nM). This intrabody bound multiple RAS mutants of all three isoforms and inhibited signaling and oncogenic transformation.^{84,85} Crystallographic

analysis revealed that iDab#6 bound the SW1 and SW2 regions to block effector interaction.⁸⁵ Despite these encouraging findings, the large size of this molecule (~25 kDa) coupled with its inability to penetrate the cell limits its effectiveness as a therapeutic agent.

Recent work from Kim and colleagues extended these findings to produce an antibody that internalized into the cell and specifically targeted oncogenic RAS to block tumorigenicity when administered systemically.³⁶ They isolated a VH fragment specific to activated RAS similar to the approach with iDab#6. This fragment was used to replace the VH fragment of a cytosol-penetrating antibody, TMab4, which they previously demonstrated entered cells through clathrin-mediated endocytosis followed by escape into the cytosol.^{86,87} The resulting chimeric IgG1, RT11, exhibited low nanomolar affinity (KD ranging from 4–17 nM) for GppNHp bound wild type and various oncogenic mutants of the three RAS isoforms. RT11 inhibited growth and signaling in RAS mutant tumor cells by competing with RAS effectors. To enhance specificity toward tumor tissues, the RGD10 cyclic peptide was fused to the N-terminus of the LC of RT11 to generate RT11-i, which was targeted to cells expressing tumor-associated integrins. RT11-i retained specificity for RAS, exhibited favorable pharmacokinetics and biodistribution in tumors, and inhibited *in vivo* growth of RAS mutant tumors in xenograft models. These results offer proof-of-principle for therapeutic targeting RAS, and potentially, other intracellular proteins using modified antibodies.

Several recent reports described high affinity synthetic binding proteins that inhibit RAS. A DARPin antibody mimetic, K27, targeted the GDP-loaded forms of wild type and oncogenic K-RAS and H-RAS, over the GTP-loaded forms with low nanomolar affinity (Figs.4 & 5D).⁸⁸ The co-crystal structure of K27 in complex with K-RAS(G12V) illustrated that the K27 binding site overlapped with that of SOS, with the bulky structure of the DARPin effectively covering the majority of the SW1 region (Fig. 5D). K27 bound GDP-loaded RAS and prevented nucleotide exchange, presumably by preventing SOS binding. Intracellular expression of K27 reduced ERK and AKT activity as well as anchorage independent growth of K-RAS(G13D) mutant colorectal cancer cells.⁸⁸

Another small protein scaffold based on the DNA binding protein sso7d, was used to generate a K-RAS(G12D) affinity reagent (Fig. 4 & 5E).⁸⁹ This scaffold reagent, termed R11.1.6, was isolated using K-RAS(G12D) and was somewhat selective for this mutant over the WT form, but only when RAS was GTP-loaded (4 nM vs 40 nM). R11.1.6 bound WT and RAS(G12D) equally well when loaded with GDP. In addition, R11.1.6 also bound with low nanomolar affinity to H-RAS and -RAS suggesting that R11.1.6 was not selective for K-RAS. Crystallographic analysis of K-RAS:R11.1.6 complexes revealed binding to SW2. This interaction reduced intrinsic GTP hydrolysis and promoted the active state structure of RAS. Despite this effect, R11.1.6 inhibited RAS:RAF interaction and reduced K-RAS(G12D)-driven MAPK activation.⁸⁹ As with other pan-RAS inhibitors, the question of toxicity remains. Furthermore, the lack of cellular penetration by this, as well as other protein-based inhibitors, relegates these compounds to experimental tools, at least until efficient intracellular delivery can be achieved.

8. RAS multimerization – a chink in the armor?

Given the challenges of isolating effective anti-RAS therapeutics, new strategies are required. Recently, renewed focus has been placed on whether RAS proteins require multimerization at the PM for their activity⁹⁰ and if these multimers take the form of dimers^{11,91,92} or nanoclusters.^{93,94} H-RAS multimers were first reported nearly 30 years ago using radiation inactivation experiments. These studies suggested that H-RAS formed dimers in cells but purified as 2–3 member multimers in solution.⁹⁵ Artificial dimerization of H-RAS in solution increased CRAF activation over monomers. Furthermore, H-RAS dimers were detectable on the PM of cells.⁹⁶ Together, these findings suggest that RAS multimerization may be important for its activity. Since this time, a variety of advanced imaging techniques have revealed the presence of -RAS, K-RAS and H-RAS dimers on membranes.^{11,91,92}

Using a combination of fluorescence lifetime imaging microscopy-fluorescence resonance energy transfer (FLIM-FRET), electron microscopy (EM) spatial mapping and fluorescence recovery after photobleaching (FRAP), Hancock and colleagues determined that ~40% of RAS proteins exist as relatively transient (0.1–1 second), 6–7 member nanoclusters that are essential for effector recruitment and activation.^{12,93,94,97} Further studies demonstrated that each RAS isoform segregated into non-overlapping GTP- and GDP-RAS nanoclusters, each containing only one RAS isoform.^{14,98} This segregation was driven by the distinct membrane targeting signals of each RAS isoform and the composition of the PM.^{98–101} However, the importance of RAS dimers vs nanoclusters has remained a point of significant debate.

The lack of a clarity in this field is likely due to the transient nature of RAS multimers (~1s),⁹³ the apparent requirement of the PM,¹¹ and lack of convincing *in vitro* data for RAS dimerization.¹⁰² Computer modeling studies suggested that dimerization may be a prerequisite step of nanocluster formation.¹⁰³ Super resolution microscopy revealed that K-RAS dimers form at physiological expression levels, monomers at lower expression levels and nanoclusters only when K-RAS was overexpressed. Further, when expressed below endogenous levels, oncogenic K-RAS activation of ERK was enhanced through artificial dimerization.¹¹ Finally, the prenylated CAAX motif was necessary and sufficient for dimer formation.¹¹ Similarly, the minimal lipid anchors of both H-RAS (residues 180–186) and K-RAS (residues 175–185) were sufficient to drive nanoclustering.^{99,100} Together, these data suggest that RAS forms dimers, rather than nanoclusters, at physiological expression levels and that these dimers are required for RAS activity. However, given that RAS levels are frequently elevated in tumors, nanoclusters may nevertheless play a critical role in RAS signaling, particular in the setting of oncogenesis.

These results explained in part the lack of dimer formation by the G-domain of RAS in solution NMR studies,¹⁰² but did not address the contribution (if any) of the G-domain to dimerization/nanocluster formation. Analysis of H-RAS crystal structures revealed a specific dimer, termed the $\alpha 4$ - $\alpha 5$ dimer, in 74 of 80 active state structures.¹⁰⁴ This dimer was not observed in inactive state RAS structures suggesting that the $\alpha 4$ - $\alpha 5$ dimer was not an artifact of crystal packing.¹⁰⁴ Similarly, N-RAS dimers were also observed in crystal

structures and through computer modeling⁹¹ These data suggested that dimerization was specific for activated RAS and conserved between isoforms, and that the allosteric lobe was key to formation of this active state dimer.

Despite the controversy over the nature and function of RAS multimers, the importance of dimerization for activation of RAF has been firmly established.^{105,106} Thus, dimerization of the associated RAS proteins may be required to promote RAF dimer formation (Fig. 6). However, this model does not necessarily require a physical RAS:RAS interaction, but may only require RAS protomers to be in sufficient proximity to drive RAF dimerization. Nevertheless, co-association of RAS at the PM may play an essential step in effector recruitment and activation.^{93,96,97,107,108}

We recently isolated a highly specific RAS monobody that provided insight into the role of RAS dimerization in signaling and oncogenic transformation.¹⁰⁴ To discover a novel modality of inhibiting RAS, we initiated an unbiased approach to inhibit RAS using monobody technology. Monobodies are single-domain synthetic binding proteins of ~95 amino acids that achieve levels of affinity and selectivity similar to antibodies⁵ yet are insensitive to the redox potential of their environment and thus can be used as genetically encoded inhibitors. We isolated a monobody, termed NS1, which bound with nanomolar affinity to H-RAS and K-RAS, but not N-RAS (Figs. 4 & 5E).^{104,109} NS1 targeted the α 4- β 6- α 5 interface in the allosteric lobe, which overlaps significantly with the proposed RAS dimer interface.^{91,104} NS1 inhibited RAF-MAPK activation by growth factor stimulation as well as oncogenic H- and K-RAS, but not N-RAS. Further, NS1 reduced oncogenic transformation by H- and K-RAS as well as impaired proliferation of RAS-mutant tumor lines.¹⁰⁴ Interestingly, NS1 primarily inhibited RAS dimerization, which in turn blocked RAF dimerization and activation.¹⁰⁴ However, further analysis uncovered additional layers of complexity to NS1's effect on RAS.¹⁰⁹ NS1 reduced K-RAS PM localization and interaction with RAF.¹⁰⁴ Comparison of the H-RAS:NS1 crystal structure with K-RAS structures indicated that NS1 may protrude toward the PM and interfere with RAS-plasma membrane orientation.¹⁰⁹ Indeed, NS1 may interfere with the interaction of the polybasic region of K-RAS with the membrane resulting in displacement of K-RAS from the membrane. Finally, NS1 reduced the GTP-bound pool of WT but not oncogenic RAS in cells.¹⁰⁹ This effect may stem from NS1 altering the orientation of the RAS G-domain with the membrane thereby perturbing interaction with SOS.¹⁰⁹ As with many RAS inhibitors, the lack of specificity for the oncogenic forms raises the question of potential toxicity issues due to inhibition of WT RAS. However, the lack of NS1 binding to N-RAS may reduce such toxicity since -RAS is sufficient to drive proliferation and maintain viability in mouse embryonic fibroblasts devoid of H- and K-RAS.¹¹⁰ Thus, a drug possessing NS1-like attributes may be less toxic than a true pan-RAS inhibitor. As with the other engineered anti-RAS affinity reagents, NS1 is not cell permeable and not suitable for cellular administration, at least with the current delivery methods.

9. RAS inhibition- is the future now?

Much progress has been made in the search for RAS inhibitors. The combined efforts of many research groups has led to the first serious discussions of whether RAS is indeed a

“druggable” target. Many approaches to achieving this goal have been outlined herein, with varying degrees of success. Despite the picomolar affinity of RAS for guanine nucleotides and high concentration of GTP in the cell, the recent success with GDP-analogues that inhibit RAS through covalently labeling of the nucleotide binding pocket demonstrates promise for this approach, at least for select RAS mutants. The chemistry of these compounds will need to be modified to optimize cell entry and minimize toxicity to enable use of such compounds *in vivo*.^{76,78} Blocking nucleotide release is unlikely to yield potent inhibitors, as most oncogenic RAS mutants have a slow rate of GTP hydrolysis.^{82,111} However, blocking the interaction of RAS with its effectors, especially RAF,^{62,64,65,89} promoting the GDP-loaded state,^{56,57,81} or blocking RAS dimerization,^{104,109} has the potential for success, if sufficient affinity and selectivity for the mutant forms can be achieved.

Compound 12 and ARS853 demonstrate significant potential for selective inhibition of oncogenic RAS and could lead to the first direct anti-RAS therapeutic, at least for tumors bearing K-RAS(G12C) mutations. Indeed, the recent success of irreversible kinase inhibitors that covalently bind Cys residues on their targets, such as Bruton's tyrosine kinase (BTK) inhibitor, Ibrutinib, and EGFR inhibitor, Afatinib,^{112–114} demonstrates the viability of such an approach. Further, there remains the possibility of taking a similar covalent labeling approach to targeting G12D and G13D mutations in RAS.⁷⁶ However, there would remain no clear path forward to inhibit other common mutations, such as G12V. therefore, isolation of inhibitors that block these mutant forms of RAS is key to isolating therapeutics to treat many RAS driven cancers. Thus, there remains much work to the development and eventual FDA approval for an anti-RAS therapeutic. However, recent advances in this area are a source of optimism that RAS is indeed a “druggable” target after all.

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Figure 1. RAS signaling pathways involved in human cancer.

A. RAS cycles between the inactive GDP-bound state and active GTP-bound state. GEFs promote nucleotide exchange whereas GAPs enhance the GTPase activity of RAS returning it to the inactive GDP-bound state. Shown are the five major RAS effect or pathways that play key roles in human cancer. B. Schematic of RAS functional domains. The sequences of the HVRs of the 4 RAS family proteins are shown for comparison. C. Annotated H-RAS crystal structure (PDB 1CRQ) with the allosteric lobe (grey), effector lobe (white), Switch 1 (SW1, yellow) and Switch 2 (SW2, tan) regions noted in colors. Regions of alpha helices and beta sheets are marked. GDP is shown in blue.

Figure 2. RAS prenylation and processing.

(1) All RAS isoforms are post-translationally modified by farnesyltransferase on the Cys of their CAAX motif. (2) Farnesylated RAS moves to the ER (endoplasmic reticulum) where the AAX is cleaved and the terminal Cys methylated. (3A) H-RAS and N-RAS move to the Golgi and are palmitoylated. (4A) Palmitoylated H-RAS and N-RAS move to the plasma membrane and other endomembranes via the secretory pathway. De-palmitoylation relocalizes H-RAS and N-RAS to the Golgi where they are re-palmitoylated and travel back to the plasma membrane. (3B) K-RAS moves directly to the plasma membrane and other endomembranes through an ill-defined mechanism. (4B) PDEδ dissociates K-RAS from all membranes and relocalizes it to the ER in an ARL-2 dependent manner.

Figure 3. Oncogenic RAS mutations increase its activity.

A. Wild type RAS is predominantly GDP loaded under basal conditions (left). Oncogenic mutations in codons 12, 13 or 61 reduce enzymatic hydrolysis of GTP to GDP, dramatically increasing the GTP-loaded pool of RAS (right). **B.** Incidence of specific H-RAS, K-RAS and N-RAS mutations in human cancer. Data acquired from the Catalogue Of Somatic Mutations In Cancer (COSMIC).⁶⁷ Percentages are based on 1,474 H-RAS, 37,642 K- RAS and 5,092 N-RAS tumor samples.

Figure 4. Current approaches to inhibiting RAS function.

RAS function has been targeted in a variety of ways such as inhibiting membrane association, targeting nucleotide exchange, disrupting effector interaction and blocking dimerization.

Figure 5. Binding interfaces of current RAS inhibitors.

Crystal structures of: A. DCAI Bound to K-RAS(WT) (PDB4DST). B. Kobe2601 bound to H-RAS(WT) (PDB 2LWI). C. Shokat's Compound 12 bound to K-RAS(G12C) (PDB 4M22). D. K27 DARPin bound to K-RAS(WT) (PDB 5O2S). E. Sso7d-based R11.1.6 bound to K-RAS(G12D) (PDB 5UFQ). F. NS1 monobody bound to H-RAS(WT) (PDB 5E95). Coloring scheme is same as in Fig. 1C except inhibitors are shown in red. Orientation of the RAS proteins is identical in each panel.

Figure 6. Proposed model of RAS activation through dimerization.

RAS cycles between GTP- and GDP-loaded states through the action of GEFs and GAPs.

Interaction of RAF with GTP-loaded RAS is not sufficient for activation. Co-association of RAS proteins promotes RAF dimer formation and activation.^{104,109}