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Review Article

Progress in targeting RAS with small molecule drugs

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RAS proteins have traditionally been deemed undruggable, as they do not possess an active site to which small molecules could bind but small molecules that target one form of oncogenic RAS, KRAS G12C, are already in preclinical and clinical trials, and several other compounds that bind to different RAS proteins at distinct sites are in earlier stage evaluation. KRAS is the major clinical target, as it is by far the most significant form of RAS in terms of cancer incidence. Unfortunately, KRAS exists in two isoforms, each with unique biochemical properties. This complicates efforts to target KRAS specifically. KRAS is also a member of a family of closely related proteins, which share similar effector-binding regions and G-domains, further increasing the challenge of specificity. Nevertheless, progress is being made, driven by new drug discovery technologies and creative science.

Background

HRAS was the first oncogene identified in human tumors. In a series of classical experiments, it was shown that a single substitution was sufficient to convert the normal protein to an oncoprotein, HRAS G12V, with powerful transforming characteristics [1]. These remarkable discoveries represented a landmark in cancer biology, with the obvious implication that drugs could be developed that target mutant proteins specifically. Thirty-seven years later, this dream is close to being realized, at least for one form of RAS oncogene, KRAS G12C. However, this may be an outlier, and indeed, clinical evaluation of drugs targeting KRAS G12C are only just beginning. Multiple additional approaches to targeting RAS are urgently needed.

RAS proteins were among the first oncoproteins to be targeted with small molecules, albeit indirectly. At a time when kinases were viewed with suspicion, the opportunity to inactivate RAS proteins by inhibiting enzymes, their prenylation seemed feasible and attractive, and, with this goal in mind, farnesyl transferase inhibitors with impressive potency and selectivity were developed and tested in the clinic [2]. Unfortunately, this approach was not successful, and attention shifted to kinase inhibitors. However, opportunities for targeting RAS proteins have increased recently, along with increased recognition of the complexity of their contributions to cancer. Here, I will review progress in targeting RAS proteins directly, with an emphasis on small molecules.

Figure 1 shows the structure of fully processed KRAS 4B. This diagram is based on the crystal structure of this protein bound to the chaperone protein PDE6-δ and represents the first structure solved of a fully processed small GTPase [3]. In the absence of PDE6-δ, the C-terminal region of KRas 4B (termed the hypervariable region, HVR), like that of other RAS proteins, is thought to be disorganized and no crystal structures are available. The G-domains of HRAS, KRAS 4A and 4B and NRAS (the first 166 amino acids) are very similar. The first 86 amino acids, which include binding sites for effectors, GEFs and GAPs, are identical. The regions of the RAS proteins that change in conformation between the inactive GDP-state and active GTP-state are Switch One and Switch Two. Figure 1 shows that these regions are very similar between close members of the RAS family, MRAS, TC21 (RRAS2), RRAS, RIT1 and RIT2. The overall topology of the G-domain is strikingly similar between all small

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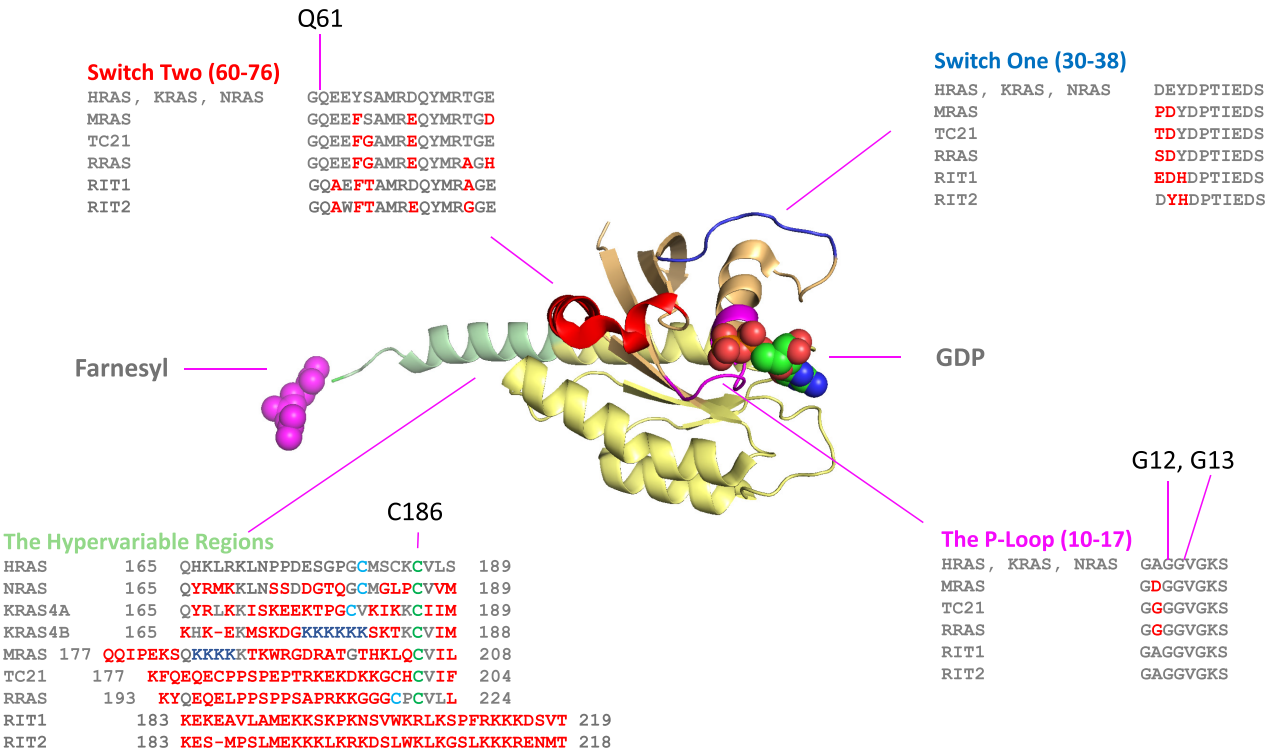

Figure 1. Structure of RAS proteins and family members.

This cartoon is derived from the co-structures of fully processed KRAS4B.GDP bound to PDE6-δ (PDB 5TAR; [3]). Sequences for the highly conserved and functionally critical Switch One and Two regions, the P-loop and hypervariable regions are shown, with divergent residues (relative to HRAS) highlighted in red.

GTPases, as well as heterotrimeric G-proteins and elongation factors [4]. Residues that comprise the GDP/GTP-binding site (K16, T35, G60, N116, K117 and A146 in HRAS) are shared between all members of the superfamily. Indeed, the first model for the structure of the human RAS proteins was based in sequence alignments with bacterial EF-Tu [5].

RAS genes are frequently activated by point mutations in cancer [6], KRAS (86% of RAS-mutant cancers), NRAS (11%) and HRAS (3%) [7]. The RAS-related proteins, RIT1, MRAS, TC21 and RRAS, that are also mutated in cancer, albeit at lower frequencies (0.2–0.3% of all cancer samples for each of these RAS relatives); www.cBioPortal.org. Tumor cells express HRAS, NRAS and KRAS genes, and all three are capable of causing malignant transformation in cells and in animal models [8]. Several theories have been proposed to account for the dominance of KRAS over NRAS and HRAS: unusual codon usage may make KRAS particularly suitable for early steps in oncogenesis [9]; KRAS has unique stem-like properties that make it more potent as an oncogene *in vivo* [10,11]; regulation of the KRAS genetic locus is more important than the gene product itself [12]; cells of origin express different RAS isoforms, even though mature tumors express them all. Regardless, KRAS has emerged as the primary therapeutic target, though HRAS was the focus of the early work on RAS biology and structure. Targeting KRAS immediately creates a difficult problem: it exists in two splice variants, each with unique biochemical properties. Oncogenic mutations occur in the conserved G-domain, so that tumors bearing these mutations are driven by two distinct oncoproteins, not one. KRAS 4B is thought to be the major isoform, based on RNA expression data primarily, but KRAS 4A is also widely expressed [13] and capable of oncogenic transformation. Interestingly, the first form of KRAS identified in Kirsten sarcoma virus was an activated form of KRAS 4A. Like Harvey sarcoma virus, this virus has an activating mutation at codon 12, and a second mutation, A59T, which creates a site for GTP-dependent autophosphorylation.

The RAS-related protein MRAS plays a unique role in cancers driven by oncogenic RAS mutations and therefore presents opportunities for therapeutic intervention. MRAS binds to SHOC2, a protein that binds PP1c and recruits this complex to the plasma membrane [14]. PP1C then de-phosphorylates P-259 on CRAF proteins

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bound to active RAS. In some cells, this is critical to RAS-dependent activation of RAF kinase (see, for example, [15]). Compounds that inhibit PP1C in this complex could therefore prevent RAS signaling in cancer cells.

While activating point mutations are the clearest indication of important roles for RAS proteins in cancer, we can infer that they are important in many cancers in which they are wild type. Loss of RAS GAPs, NF1 and its associated proteins SPRED1, RASA1 and RASA2, through mutation or deletion, occur frequently in many cancers [16]; in these cancers, multiple members of the RAS family are likely activated, though precisely which ones are most important is hard to determine. In breast cancer, RASAL2, another RAS GAP, is suppressed epigenetically, again leading to hyperactivating of multiple types of RAS protein [17]. Proteins involved in activating RAS are also mutated in human cancers, including SOS1 and SOS2 [18]. Wild-type RAS proteins are also amplified in tumors (KRAS 2% of all tumors, NRAS 0.3% and HRAS 0.3%): (http://www.cbioportal.org/). Finally, many tumors are driven by misregulated tyrosine kinases, which signal through wild-type RAS proteins [16].

Validating RAS proteins as therapeutic targets

It is important to establish to what extent clinically relevant tumors are dependent on RAS for their growth or survival. Analysis of tumor DNA at various stages of progression reveals that most (but not all) pancreatic ductal adenocarcinoma [19] and lung adenocarcinoma [20] tumors are clonal with respect to KRAS mutations, suggesting that these mutations initiate the disease and remain as drivers throughout tumor evolution including metastatic lesions. Targeting KRAS in patients suffering from these diseases therefore seems like a promising strategy. In squamous carcinoma of the lung [20] and in colorectal cancer [21], KRAS mutations appear later, raising the concern that these diseases might be polyclonal with respect to KRAS. In AML, NRAS mutations are often not clonal. Indeed, early analysis of Ras expression in these leukemias revealed the first evidence of sub-clonal oncogenic Ras mutations [22]. As technology continues to improve, it is likely that analysis of tumor evolution through deep sequencing will give provide a clearer picture of the role of RAS genes and passengers in tumor evolution.

Significant efforts have been directed at validating KRAS proteins as targets in tissue culture systems. KRAS genes have been knocked down using siRNA, shRNA and CRISPR in multiple cell lines derived from different types of cancer. In one highly cited study, Singh et al. [23] reported that cell lines with mutant KRAS varied considerably in their dependence on KRAS for survival. This study focused on induction of apoptosis as a key measure of dependence and reported that cell lines that were more differentiated in character were more likely to die than those that had underdone epithelial-to-mesenchymal transition. In a more recent study, we confirmed that cell lines fell into two major categories in which the KRAS-dependent set was more epithelial-like than mesenchymal cells, though other parameters such as dependence on RAF/MAPK signaling and on glycolytic pathways were additional distinguishing features. The KRAS-independent set had switched oncogene addiction from KRAS to RSK and mTOR, and depended on oxidative phosphorylation rather than glycolysis. We also identified a minor subset that depended strongly on RAL and PI 3′-kinase signaling. Furthermore, we found that pancreatic cancer cell lines were generally more frequently dependent on KRAS than cells derived from lung adenocarcinoma [24]. This latter correlation can also be seen in the DepMap database (https://depmap.org/portal/), which assigns every gene a 'dependency score' across large panels of cells, interrogated by siRNA, shRNA or CRISPR, by several different organizations. Overall, these studies show that many KRAS-mutant cell lines retain dependence on KRAS in cell culture, while many others are relatively independent (see, for example, [25]), and no single biomarker can predict which category a cell line will fall into. Furthermore, KRAS dependency depends on growth conditions and whether cells are grown in 2D or 3D and on the definition chosen for dependence. These caveats underscore a major limitation in understanding KRAS as a target: we do not know which aspect of KRAS biology is most relevant in human cancer.

In mouse models of cancer, the conclusion is clearer, though far fewer situations have been analyzed. When RAS oncogenes are ablated in tumors, dramatic regressions are generally observed (reviewed in ref. [26]). In one notable study, surviving clones emerged through amplification of the YAP1 locus [27]. While escape mutations are expected in any targeted therapy, the majority of data from *in vivo* studies support targeting KRAS directly for cancer therapy.

Targeting Ras through preventing farnesylation

When RAS proteins were shown to be modified by farnesylation [28], and that this modification is essential for biological activity [29,30], extensive efforts were launched find compounds that block farnesyl transferase, using

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small molecules, peptide mimetics and natural products as potential sources of lead compounds (reviewed in ref. [2]). Indeed, HRAS proteins appear to be excellent targets for farnesyl transferase inhibitors. Early studies in cells and in mice showed dramatic responses to these compounds, without serious side effects. This safety profile may be attributed to the fact that HRAS is dispensable in mice. Nevertheless, the lack of overt toxicity of farnesyl transferase inhibitors was surprising, as more than a hundred other proteins are farnesylated and presumably depend on farnesylation for their activity [31]. For example, the structural protein prelamin A is farnesylated, and this modification is essential to transport prelamin A to the nuclear pore and into the nucleus where prelamin A is cleaved to become lamin A, and an essential part of nuclear integrity. In the disease of premature ageing, progeria, mutations in the prelamin A gene create a protein that can be farnesylated, but not cleaved, leading to accumulation of the precursor and abnormal nuclear structures [32]. Farnesyl transferase inhibitors have shown encouraging signs of clinical activity in this form of progeria. 'Experimental Drug Is First To Help Kids With Premature-Aging Disease'. *NPR*, 24 September 2012.

Recently, clinical evaluation of the farnesyl transferase inhibitor tipifarnib has been initiated in patients pre-selected for HRAS mutations, and early data suggest clinical activity, particularly in squamous cells head and neck cancer (https://kuraoncology.com/pipeline/#tipifarnib). If confirmed, this would obviously be a good option for patients suffering from these malignancies and other malignancies in which HRAS is mutated. For example, Fagin and colleagues recently showed that tipifarnib inhibits HRAS-driven dedifferentiated thyroid cancers in mouse models. They also noted instances of emergent resistance, through loss of NF1 or activation of the G-protein subunit GNAS [33]. HRAS is also mutated in bladder cancer at ~5% frequency. Indeed, RAS mutations were first detected in the human bladder cancer cell line EJ/T24. Clinical responses to tipifarnib suggest that targeting RAS proteins may be an effective strategy, even as a single agent. Unfortunately, KRAS4A, KRAS4B and NRAS proteins can be prenylated by geranylgeranyl transferase following farnesyl transferase inhibition. Therefore, unlike HRAS, tumors driven by these oncoproteins failed to respond to farnesyl transferase inhibitors [2].

Following farnesylation, the C-terminal three amino acids of all RAS proteins are removed by the enzyme RCE1 (RAS-converting CAAX endopeptidase 1), and the exposed carboxyl acid is carboxymethylated by ICMT (isoprenylcysteine carboxymethyl transferase) [2]. These modifications are necessary for RAS activity, but their potential value as drug targets for RAS-driven cancers is unclear, largely because they act on multiple substrates, even beyond the RAS superfamily. Nevertheless, it is interesting that a screen for genes that are essential for AML cells driven by mutant RAS, relative to those driven by wild-type RAS, revealed ICMT and RCE1 as top hits, alongside RAF1 and SHOC2 [15]. Furthermore, analysis of gene dependencies across multiple cell lines (https://depmap.org/portal/) revealed that dependency on RAF1 correlated with dependency on RCE1, again suggesting that this enzyme is critical to RAS function. KRAS 4A, NRAS and HRAS are palmitoylated: inhibiting palmitoylation presents another possible approach to blocking function of these proteins, though this has not effectively explored. Over 2000 mammalian proteins are palmitoylated, however, making specificity a serious challenge [34].

Targeting mutant alleles

Ideal anti-RAS drugs would target mutant forms specifically. This dream is on the point of being realized. In 2013, Shokat and colleagues identified compounds that bind covalently and specifically to KRAS [35]. About 34% of KRAS mutations in lung adenocarcinoma are G12C [6,36], presumably because the transversion that generates the KRAS G12C protein is typical of carcinogens in tobacco smoke [37]. Compounds were identified using an approach referred to as tethering, in which a library of compounds with thio-reactive warheads are mixed with a target protein (the G-domain of KRAS G12C in this case) under reducing conditions, as described in Figure 2 and reviewed by Erlanson et al. [38]. Compounds that react with the target cysteine remain covalently bound under these conditions if they also bind non-covalently at a nearby site. In this case, the non-covalent site was identified under Switch Two, a previously unrecognized site that is only accessible in the GDP-bound form of the KRAS G12C protein. At first, this preference for GDP seemed to be a potential disadvantage, as oncogenic mutants, including KRAS G12C [39], are predominantly in the GTP-bound forms at steady state. Fortunately, the intrinsic GTPase activity of KRAS G12C is relatively high, so that the protein cycles between the GTP-bound and the GDP-bound states sufficiently frequently to make it vulnerable to attack before is transitions back to the GTP-state.

Lead compounds were developed further by Wellspring Biosciences, who showed that compounds ARS-853 and ARS-1620 (Figure 3) inhibit KRAS G12C effectively and specifically in cells and in animals [39]. One

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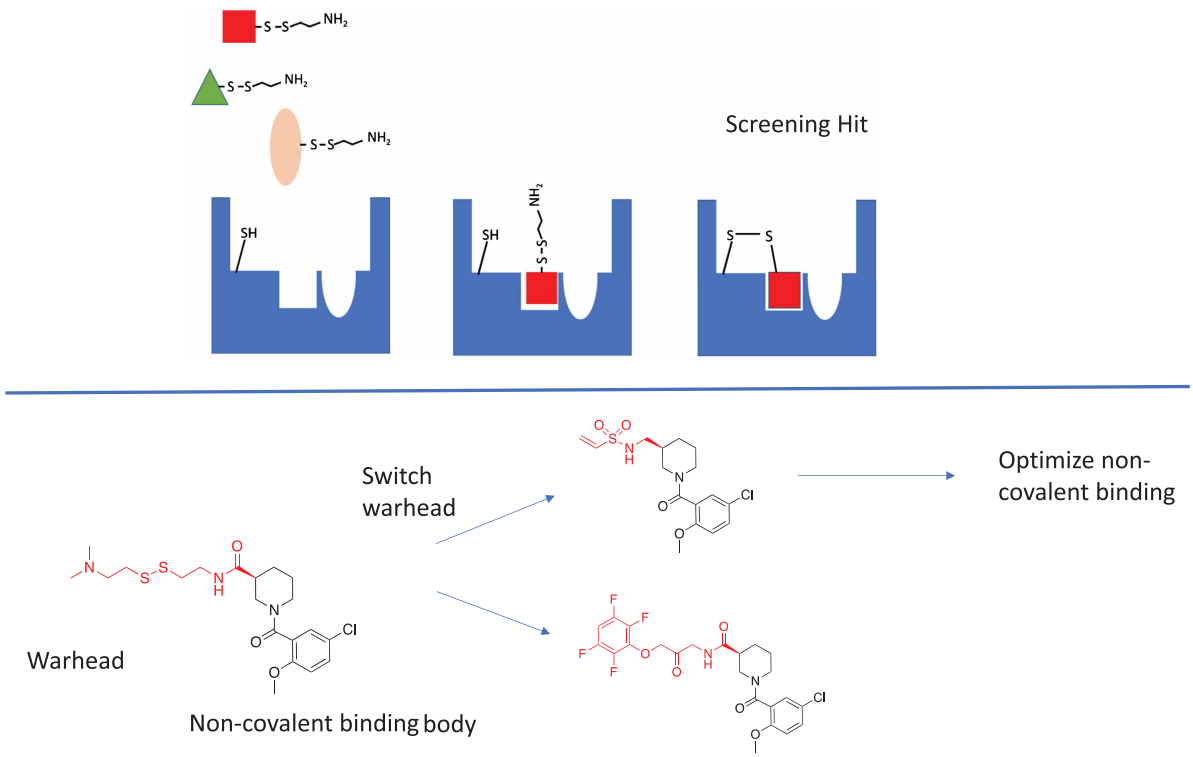

Figure 2. Identifying compounds that bind covalently using tethering.
In the cartoon, an SH-group represents a reactive cysteine residue. The target protein is exposed to a library of thio-reactive compounds under reducing conditions. Compounds bind, generally with low affinity, and then react covalently with the adjacent cysteine. After selecting a hit compound based on its efficiency of labeling, detected by mass spectrometry, the warhead is switched to an irreversible electrophilic group, and the non-covalent body of the hit compound is modified to improve binding and selectivity [38]. An example from the UCSF tethering library is shown.

potential challenge of the tethering approach is that compounds that bind with low affinity at the non-covalent site need to be armed with a reactive warhead to engage their target cysteine before they dissociate. Reactive warheads can potentially react promiscuously with other reactive proteins and compounds in cells, including glutathione. However, recent analysis of the mechanism of action of ARS-853 and ARS-1620 revealed an unexpected feature that enables these compounds to engage their target effectively, despite very low affinities for the non-covalent-binding step, and relatively weak electrophilic warheads: when the drug binds with low affinity to the induced pocket under switch two, it is well positioned to react covalently with Cys-12: this positioning greatly accelerates the rate of reaction. In addition, an adjacent lysine residue, Lys-16, stabilizes the transition state, thereby accelerating the reaction [40]. By exploiting the correct positioning of the drug, and taking advantage of local residues near the pocket, the protein effectively catalyzes the reaction and facilitates use of less reactive warheads as inhibitors.

Several groups have now developed KRAS G12C inhibitors though few details have been published. The first KRAS G12C inhibitor to enter clinical trials is Amgen 510 (https://clinicaltrials.gov/ct2/show/NCT03600883). Recently, a new class of G12C compounds was identified, using *in silico* docking rather than empirical screening. These compounds destabilize KRAS G12C and increase nucleotide exchange, in contrast with the compounds described recently [41]. In addition, Gray and colleagues reported a GDP analog to which an electrophilic warhead had been attached: this compound covalently modifies Cys-12 in KRAS G12C and shows the potential of leveraging nucleotide binding to irreversibly block KRAS function [42].

Identifying drugs that bind non-covalently to KRAS

While the cysteine substitution in KRAS G12C is chemically reactive, other common substitutions, such as G12D, are even more challenging [43], and G12V and other alleles will have to be targeted using different

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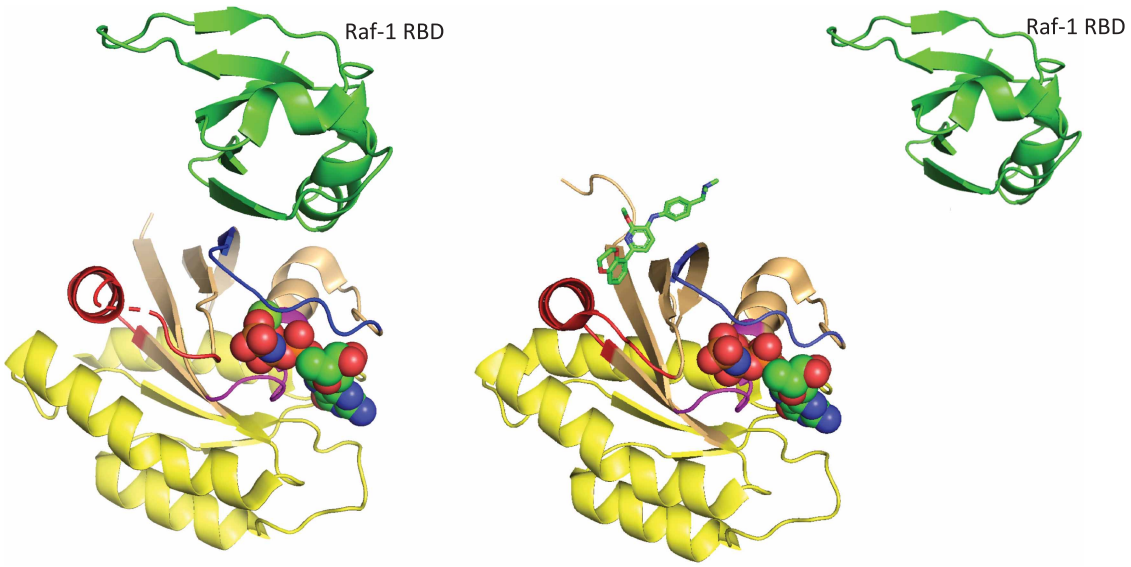

Figure 3. KRAS4B.GDP bound to either ARS-1620 under Switch Two, or a small molecule identified by NMR-based screening, bound at the SOS-binding site.

The cartoon is based on PDB structure 5V9U, from Wellspring Biosciences [69], with the small molecule fragment (2-hydroxyphenyl)(pyrrolidin-1-yl)methanethione from PDB: 4EPT from Fesik and colleagues [49] added for comparison.

approaches. Alternatively, drugs could be developed that bind to KRAS without distinguishing between wild-type and mutant forms. These compounds would have to spare HRAS and/or NRAS to be safe and tolerable. Ablation of KRAS in adult mice was relatively well tolerated, as discussed elsewhere [8]. Nevertheless, identifying compounds that bind selectively to KRAS specifically will be difficult. The similarity between the G-domains of these proteins and of RAS-related proteins RRAS, RRAS2 and MRAS (Figure 1) makes this extra challenging. Targeting the HVR of KRAS offers opportunities for selectivity, but since this comes in two varieties, KRAS 4A and KRAS 4B, it does not simplify the problem.

SOS-binding pocket

Early efforts to design compounds that bind to RAS proteins based on crystal structures and computer algorithms led to the identification of compounds that bind near Switch Two and inhibit SOS-mediated nucleotide exchange. These and other earlier RAS-binding compounds have been reported, but have not been developed further [44,45] (reviewed recently in refs [46,47]). New technologies have facilitated efforts to find compounds that bind to RAS proteins, despite the lack of deep binding pockets. These include 'SAR by NMR', a powerful technique developed by Dr Stephen Fesik and colleagues [48], which led to compounds that bind to RAS, again at the SOS-binding site (Figure 3). These compounds bind with low affinity, but nevertheless were among the first compounds published as RAS binders and verified by NMR and X-ray crystallography (Figure 2) [49,50]. Interestingly, two groups have succeeded in making peptides that bind to Ras proteins directly: like the compounds described above, these peptides bind near Switch Two and prevent interaction with GEFs such as SOS [51,52] and more recently, antibody mimetics referred to as darpins have been identified that bind to RAS proteins with a K_d of 4 nM and block GDP/GTP exchange [53]. Unfortunately, compounds that inhibit SOS binding are expected to inhibit normal RAS function, but have little effect on oncogenic mutants.

Effector-binding region

More recently, Stockwell and colleagues described compounds that bind to RAS, designed *in silico* using Glide SP software (Schrodinger), focused on three shallow pockets around residues 32, 38 and 59, in Switch One and adjacent to Switch Two. The site at which these compounds dock is identical between all forms of RAS, and they showed activity in cell systems and in mouse models [54]. Whether these specific compounds are

developed further remain to be seen, but this approach is likely to be used more frequently in the future as more structures are solved and software and algorithms are developed further.

Antibodies that bind to RAS proteins have been used to block their function in living cells. Stacey and colleagues [55] micro-injected neutralizing antibodies to establish several key aspects of RAS signaling, including RAS' role in cell cycle progression, and the relationship between RAS and Raf kinase. In collaboration with Dr James Feramisco, we showed that antibodies specific for G12S or G12R RAS oncoproteins reversed transformation of cells driven by cognate RAS alleles [56]. Rabbitts and co-workers [57], Roberts and co-workers [58] and others have developed intracellular antibodies or monobodies [59] that neutralize RAS activity in cells and *in vivo* [60]. These strategies have not yet translated into preclinical compounds. However, Quevedo et al. [61] have used intracellular antibody fragments to develop a powerful screen for small molecules that bind to RAS in a competition-based screen. Interestingly, the first hits from this screen bound at the familiar SOS pocket, but structure-guided chemistry efforts led to compounds that bind directly close to Switch One, the effector-binding site, with sub-micromolar affinities, making these among the most potent non-covalent binders reported to date (Figure 4).

Another innovative screen led to the identification of compounds that prevented KRAS-dependent activation of BRAF kinase in the presence of phosphatidyl serine [62]. One of these compounds was later shown to bind to KRAS and stabilize its orientation in the membrane in such a way that effector engagement was occluded [63].

Targeting KRAS recycling

KRAS proteins do not turn over rapidly, if at all, but there is evidence that they cycle on and off membranes, though mechanisms regulating binding and release from the plasma membrane are relatively poorly understood. For KRAS 4B, phosphorylation of Ser-181 affects membrane association [64], as does binding to calmodulin [65,66], but the significance of these processes is still being analyzed (see ref. [11] for example). Recently, another mode of regulating KRAS localization was described. PDE6-δ binds to processed KRAS and enables it to recycle from the plasma membrane to endosome and back to the plasma membrane. Compounds that prevent KRAS binding to PDE6-δ were shown to prevent KRAS localization in the plasma membrane and so prevent KRAS signaling [67,68]. These compounds were therefore potential lead compounds in a new approach to KRAS targeting.

Recently, the structure of full-length, processed KRAS 4B bound to PDE6-δ was solved [3]. Several interesting and unexpected features were revealed, including the mechanism by which PDE6-δ could accommodate

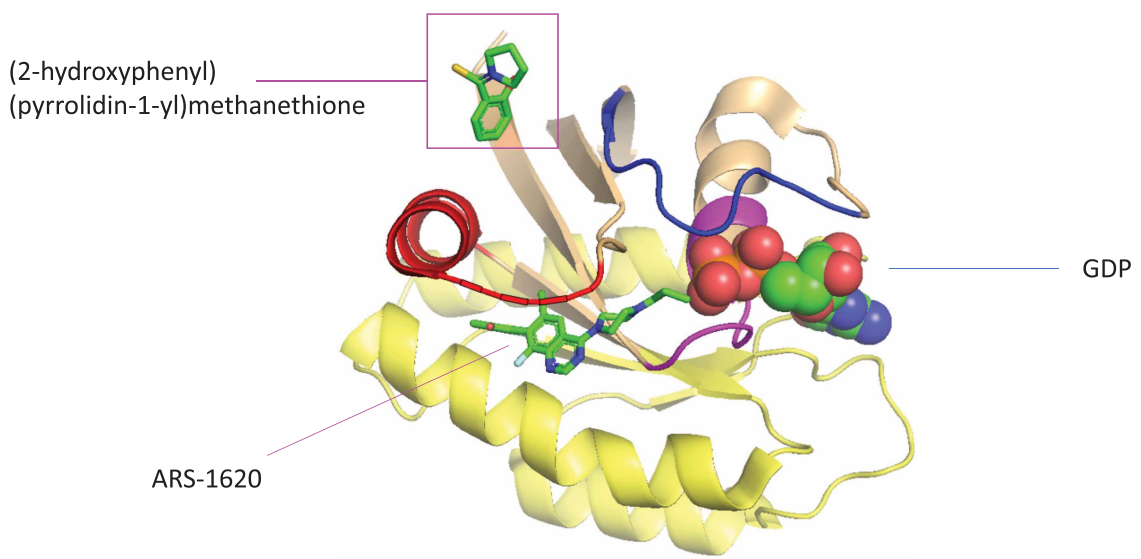

Figure 4. Structure of Ras bound to RAF RBD or to compound Abd-7 at the effector-binding site.

The cartoon is based on PDB 6FA4, from Rabbitts and colleagues [61] (right panel). The structure on the left panel shows HRAS.GppNHp bound to the Ras-binding domain of Raf1 (PDB: 4G0N) from Mattos and colleagues [70].

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either farnesylated KRAS 4B or geranylgeranylated KRAS 4B, and the basis of selectivity for KRAS 4B over 4A or other RAS family members. The specificity of PDE6-δ for KRAS 4B explained a previously troubling aspect of PDE6-δ biology: mouse knockouts of PDE6-δ are viable, whereas knockouts of KRAS are not. This would argue against an essential role for PDE6-δ in KRAS biology. However, it now seems likely that PDE6-δ is specific for the KRAS 4B isoform, so that inhibitors might block function of this protein, but spare KRAS 4A and allow normal mouse development.

Summary

Compounds that bind directly to RAS proteins, covalently or non-covalently, have been discovered, through the application of tethering, NMR-based fragment screening, *in silico* modeling and by various creative screening approaches. Of these approaches, direct attack on KRAS G12C is currently by far the most promising, but the challenge of targeting other mutants, or KRAS selectively, is fueling research efforts, including the NCI-supported Ras Initiative at the Frederick National Laboratory for Cancer Research. KRAS has been validated as a drug target and the clinical need remains enormous. No obvious unsurmountable barriers exist, though execution will be extremely difficult. Progress in the last few years has been remarkable, and there are reasons to be optimistic.

Abbreviations

HVR, hypervariable region; ICMT, isoprenylcysteine carboxymethyl transferase; RCE1, RAS-converting CAAX endopeptidase 1.

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Competing Interests

F.M. is a consultant for Leidos Biomedical Research, Inc., Amgen, Inc., Daiichi Sankyo Company, Limited and Pfizer, Inc. He is also Co-founder of BridgeBio Pharma LLC and Araxes Pharma LLC.

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