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Targeting Mutant KRAS for Anticancer Therapeutics: A Review of Novel Small Molecule Modulators

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Abstract

The RAS proteins play a role in cell differentiation, proliferation, and survival. Aberrant RAS signaling has been found to play a role in 30% of all cancers. KRAS, a key member of the RAS protein family, is an attractive cancer target, as frequent point mutations in the KRAS gene render the protein constitutively active. A number of attempts have been made to target aberrant KRAS signaling by identifying small molecule compounds that (1) are synthetic lethal to mutant KRAS, (2) block KRAS/GEF interactions, (3) inhibit downstream KRAS effectors, or (4) inhibit the post-translational processing of RAS proteins. In addition, inhibition of novel targets outside the main KRAS signaling pathway, specifically the cell cycle related kinase PLK1, has been shown have an effect in cells that harbor mutant KRAS. Herein we review the use of various high-throughput screening assays utilized to identify new small-molecule compounds capable of targeting mutant KRAS-driven cancers.

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INTRODUCTION

The RAS (rat sarcoma) protein family members are all low-molecular-weight GTP-binding proteins that play a role in regulating cell differentiation, proliferation, and survival.^{1,2} There are three main members of the RAS family: HRAS, NRAS, and KRAS, all of which have been found to drive cancer formation and progression.^{1–3} Mutations in RAS are found in approximately 30% of human cancers.² In the absence of a RAS mutation, increased RAS activity in human tumors has been shown to be the result of gene amplification, overexpression, or increased upstream activation.⁴ Single point mutations of the RAS gene, affecting residues G12 and G13, abolish GAP-induced GTP hydrolysis through steric hindrance, while mutations of residue Q61 interfere with the coordination of a water molecule necessary for GTP hydrolysis.⁵ These mutations render the protein constitutively active, and the persistence of active GTP-bound RAS leads to the constant activation of its downstream effector pathways. KRAS is the most frequently mutated RAS isoform, having been shown to be mutated in 90% of pancreatic adenocarcinomas, 45% of colorectal cancers, and 35% of lung adenocarcinomas.^{2,6} KRAS mutations have been associated with increased tumorigenicity and poor prognosis.⁴ Additionally, the inhibition of activated RAS has been shown to revert malignant cells to a nonmalignant phenotype and cause tumor regression both in vitro and in vivo.^{4,7,8} Thus, KRAS is an attractive therapeutic target for a number of cancers.

While the development of a small molecule inhibitor of the constitutively active KRAS protein would be ideal as a cancer therapeutic, 25 years of work on drugs targeting the GTP binding pocket of mutant KRAS have thus far proven to be unsuccessful. To date, no effective therapy that specifically targets mutant KRAS is available. Targeting the constitutively active molecular switch of KRAS is quite difficult because the role of GDP or GTP is to stabilize inactive or active states of the RAS protein.⁹ This is in contrast to protein kinases in which phosphoryl transfer from ATP to a substrate is a rapid, catalytic process.⁹ Additionally, because of picomolar affinity between KRAS and GTP, as well as the micromolar concentration of GTP in the cell, a competitive inhibitor is not particularly feasible.⁹ Furthermore, KRAS activation and signaling are accomplished through protein–protein interactions (PPIs) with guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and the various KRAS effector proteins.¹⁰ PPIs are challenging to target because of the relatively featureless topologies of the surfaces involved.¹¹ In spite of these issues, a number of attempts have been made to target aberrant KRAS signaling at different levels.

KRAS SIGNALING PATHWAY

Gaining a comprehensive understanding of the life cycle of the KRAS protein is essential to mounting a successful drug discovery effort. After RAS proteins are translated, they must undergo a series of post-translational modifications (Figure 1).^{2,12–14} Initially, the thiol group of the terminal cysteine in KRAS is farnesylated by farnesyltransferase (FTase).^{15–18} Next, the CAAX (C = cysteine; A = isoleucine; X = serine or methionine)^{19,20} protease RAS-converting enzyme 1 (RCE1) cleaves the terminal AAX amino acids, and the carboxy group of the cysteine is methylated by isoprenylcysteine carboxymethyl-transferase 1

(ICMT1).^{21–23} Finally, palmitoyl transferase (PTase) transfers a palmitoyl moiety to cysteine residues located just upstream of the C-terminus of HRAS, NRAS, and the KRAS-4A isoform.³ The RAS protein then forms a stable interaction with the cell membrane via its farnesyl and palmitoyl groups as well as the positively charged C-terminal lysine residues. Thus, the post-translational modification of KRAS offers several possible drug targets: (1) FTase,²⁴ (2) CAAX endopeptidase,^{25,26} (3) methyltransferase,²⁷ and (4) palmitoyl transferase.¹⁶

RAS proteins cycle between the inactive GDP-bound form and the active GTP-bound form. This activation state is regulated by GEFs and GAPs (Figure 2).^{6,28–33} When a growth factor binds to a tyrosine kinase receptor, it forms an active homodimer that undergoes autophosphorylation. This leads to binding of the adaptor proteins SHC and growth factor receptor bound 2 (GRB2). GRB2 then associates with GEFs through its SH3 domain.³⁴ GEFs exchange bound GDP for GTP, thereby activating the RAS protein and initiating a signaling cascade. To terminate signaling, GAPs stimulate the intrinsic GTPase activity of RAS proteins, causing the hydrolysis of GTP to GDP, leading to deactivation of the RAS protein. Thus, upstream targets for KRAS inhibition include (1) tyrosine kinases,³⁵ (2) the GRB2/GEF interaction,³⁶ and (3) GEFs.³⁷

KRAS has many downstream effector proteins that it can interact with to alter cell survival and proliferation (Figure 3).^{3,37,38} Active GTP-bound KRAS predominantly signals through RAF protein kinases, phosphoinositide 3-kinases (PI3K), guanine nucleotide exchange factors for the RAS-related protein Ral (RalGDS), and phospholipase C ϵ (PLC ϵ). RAF initiates the mitogen-activated protein (MAP) kinase cascade, which activates extracellular signal-regulated kinase (ERK). This active kinase has numerous targets, including the transcription factor ELK1, which regulates the expression of cell-cycle progression genes.^{39–43} The PI3K pathway activates AKT and leads to the transcription of prosurvival genes, cytoskeletal remodeling, and the activation of numerous transcription-factor pathways, most notably NF- κ B.^{44,45} RalGDS activation leads to the activation of the RAL1 binding protein: this in turn leads the inhibition of FOX transcription factors⁴⁶ which are involved in cell growth, proliferation, and differentiation. RalGDS can also signal through the JNK pathway to stimulate the transcription of prosurvival and cell-cycle progression genes.^{47,48} The activation of PLC ϵ results in protein kinase C (PKC) activation and the mobilization of calcium from intracellular stores.⁶ It is clear from this brief overview that there are numerous downstream targets that can be inhibited in order to try and overcome aberrant KRAS signaling.

HIGH-THROUGHPUT SCREENING

The screening of chemical compounds for pharmacological activity has been ongoing in various forms for the past 40 years. The recent advances in molecular biology, computer science, robotics, instrumentation, and overall process engineering have established high-throughput screening as a predominant tool in the field of drug discovery to identify compounds that are active against various biological targets or phenotypes. High-throughput screening (HTS) has been used to identify structurally diverse chemical compounds that are

capable of blocking different stages of KRAS signaling in order to inhibit the proliferation of mutant KRAS-driven cancer cells both in vitro and in vivo.

Synthetic Lethality Screening

Synthetic lethality screening aims to identify small molecules that selectively kill oncogene-expressing engineered cell lines.⁴⁹ Different groups have performed HTS screening, using different cell line models, in an attempt to find compounds that are lethal to mutant KRAS-driven cell lines. A few such examples and their successes are discussed below.

Torrance et al. utilized DLD-1 colon cancer cells that harbor mutant KRAS and an isogenic derivative in which the mutant KRAS allele was deleted (KO cells).⁵⁰ A yellow fluorescent protein expression vector was introduced into the DLD-1 cells, while a blue fluorescent protein was introduced into the isogenic derivative. Equal volumes of the two cell lines were cocultured and tested against 29 440 compounds from the ChemBridge and NCI libraries, and results were assessed by analyzing the differential fluorescence intensity for blue and yellow fluorescent protein. Positive hits were then screened as above but over a range of drug concentrations. The main hits from this screen were a sulfinyl cytidine (**1**) derivative (**2** or **3**) and triphenyltetrazolium (**4**) (Figure 4).⁵⁰ A mixture of the isomers **2** and **3** on DLD-1 and KO cells demonstrated IC₅₀ values of 125 and ~750 ng/mL, respectively, while the IC₅₀ values for **4** on DLD-1 and KO cells were ~2 and ~12 µg/mL. Both compounds demonstrated a 6-fold selectivity for the mutant KRAS-cell-lines-containing DLD-1 cells over the KO cells. Further research demonstrated that the mixture of isomers **2** and **3** selectively inhibited the growth of the KRAS^{G12V}-transformed rat kidney epithelial cells compared to the parental RK3E cell line, while compound **4** had no effect in these two assays. The antitumor activity of the mixture of isomers **2** and **3** was also assessed in vivo using HCT116 and DLD-1 xenograft models, both of which harbor a single G13D point mutation in the KRAS gene; at 150 mg/kg, tumor volumes were reduced by approximately 65% and 45%, respectively.

Guo et al. utilized human ovarian surface epithelial cells that were immortalized with human telomerase reverse transcriptase (hTERT) and SV40 (T29) transformed with either mutant KRAS (T29Kt1) or mutant HRAS (T29Ht1).⁵¹ Ten-thousand compounds from the ChemBridge library were screened against the three cell lines, and a sulforhodamine B (SRB) colorimetric assay was used to determine cytotoxicity. Initial hits found to inhibit >50% of cell growth were confirmed by two more SRB dose-response tests. The hit identified was oncrasin 1 (**5**; Figure 5). Compound **5** induced cytotoxicity in T29Kt1 cells in a dose-dependent manner, with an IC₅₀ of 4.81 µM. However, no cytotoxicity was detected in T29 or T29Ht1 cells. Further research demonstrated that **5** effectively killed KRAS mutant lung cancer cells (H460, H2122, H2887, and A549) with an IC₅₀ of 3 µM but had little effect on the cell viability of lung cancer cells with wild-type RAS (H322 and H1395) or lung cancer cells that harbor mutant NRAS (H1299 and H2087). These results illustrate that **5** is highly selective for cells transformed with mutant KRAS. The in vivo antitumor activity of **5** was assessed using an H460 lung cancer xenograft model with daily intraperitoneal (ip) injections of 100 mg/kg. Compound **5** inhibited tumor growth by 75.4% compared to a solvent control, prolonged survival from 24 to 32 days and was well tolerated

as evidenced by no weight loss. Additionally, the researchers noted that **5** was also synthetically lethal to protein kinase C α ; but the biological mechanisms behind this dual synthetic lethality remain to be determined.⁵¹

Shaw et al. utilized mouse embryonic fibroblasts (MEFs) that expressed oncogenic KRAS^{G12D} to identify effective therapeutic agents for patients with KRAS mutant tumors.⁵² Over 50 000 compounds were screened against KRAS wild-type and mutant expressing MEF cell lines using a high-throughput ATP-based cell viability assay (CellTiter-Glo (Promega)). This assay identified toperisone (**6**; Figure 6) as a lead, which showed significant differential activity in the KRAS WT cell line compared to the KRAS^{G12D} cell line in the viability assays, as well as in a bromodeoxyuridine (BrdU) incorporation assay. Subsequently, 10 derivatives of **6** were examined to explore structure–activity relationships. The derivative lanperisone (**7**; Figure 6)⁵² showed the most potent and selective activity against KRAS^{G12D} MEFs, with an IC₅₀ of 4 μ M in an ATP-based cell viability assay. Compound **7**; a single isomer with a minor structural modification from **6**; was shown to induce reactive oxygen species that are inefficiently scavenged in KRAS mutant cells leading to apoptosis. This increase in oxidative stress is the putative mechanism of action for compound **7**. In vivo pharmacological studies were carried out using compound mutant MEFs carrying KRAS^{G12D} in a p53-null background. Results showed that tumors from mice treated with **7** were 24% smaller (by volume) and 37% smaller (by weight) compared to tumors from control animals. With human safety and pharmacokinetic data already completed, **7** is poised for rapid clinical development.

In summary, researchers have identified several lead compounds to treat KRAS driven cancer using synthetic lethality screening. However, it should be noted that the synthetic lethality screening approach is based on a phenotype, not a molecule target. Thus, while there is selectivity based on the expression of mutant KRAS, the exact target at a molecular level is unknown. This fact can limit the full understanding of the biology involved in the selectivity of the compounds and accordingly the design of more druglike analogues.

Blocking KRAS/GEF Interactions

While PPIs are challenging to target because they often lack a defined binding pocket for small molecules, fragment-based screening is well-suited for this endeavor.¹¹ Outside the guanine nucleotide-binding domain, the KRAS protein has no known allosteric sites for potential modulation.⁵³ Several RAS-GEFs have been identified, and most research efforts have been focused on son of sevenless (SOS). The pockets adjacent to the KRAS/SOS contact surface are amenable to small-molecule binding.⁵³ In 2012, two groups at Genentech and Vanderbilt independently utilized NMR-based fragment screening to identify small molecules that bind to the pocket adjacent to the KRAS/SOS interaction surface and subsequently interfere with the KRAS/SOS interaction.^{10,53}

Fragments are defined as low molecular weight (120–250 Da), moderately lipophilic (clogP < 3), highly soluble organic molecules that typically bind to their target protein with low affinity (100 μ M to millimolar).^{54,55} In order to identify fragments that bind to KRAS^{G12D}, both groups utilized a target-based NMR method, measuring the changes in the chemical shift of a labeled target protein upon ligand binding, with ¹⁵N-labeled GDP-bound

KRAS^{G12D}. Fragment binding was assessed by changes in the resonance position of cross-peaks in the ¹H/¹⁵N two-dimensional heteronuclear single-quantum coherence spectra.^{56,57} The Genentech group also used the ligand-based approach of saturation transfer difference.⁵³ In this approach, the protein is selectively irradiated and magnetization is transferred from the protein to the bound ligand. The magnetized ligand is then detected in the free solution, and difference spectroscopy (with an unirradiated protein reference spectrum) is utilized to identify the bound ligand. Although these methods are not considered to be a high-throughput methodology, NMR automation and the pooling of test fragments for screening allowed for large fragment libraries to be screened relatively quickly.

DCAI (**8**; Figure 7a), identified by Genentech, was found to bind in the hydrophobic pocket located between the α 2 helix (of the switch II region) and the core β -sheet of the KRAS protein and thereby interrupt KRAS/SOS association.⁵³ Titration studies with **8** indicated that it blocked both nucleotide exchange and release reactions, with IC₅₀ values of 342 ± 22 and 155 ± 36 μ M, respectively. It was also noted that a secondary electronegative binding cleft is formed in the protein upon fragment binding. It is proposed that **8** blocks the first phase of the exchange reaction by inhibiting nucleotide release from KRAS.

The Vanderbilt group¹⁰ screened 11 000 fragments of which 140 were shown to bind to GDP-bound KRAS^{G12D}. Indole derivative **9** (Figure 7a) is a representative example of the hits that exhibit binding affinity to KRAS^{G12D} at 1.3–2 mM. Furthermore, compound **9** was shown to inhibit SOS-mediated nucleotide exchange, thereby preventing the activation of KRAS. Using structure-based design, the Vanderbilt group added functionality to their original fragment hit in order to target the secondary electronegative binding cleft formed upon fragment binding. The resulting *S*-isoleucin derivative **10** and D-proline analogue **11** (Figure 7a)¹⁰ displayed improved binding affinity (190 and 340 μ M, respectively) and were capable of inhibiting a greater percent of SOS-catalyzed nucleotide exchange. Additionally, a molecular modeling overlay indicates that compound **11** binds to the same hydrophobic pocket as **8**; however, they appear to make very different contacts (Figure 7b).⁵⁸

Inhibiting KRAS Effectors

Because of the difficulty of targeting KRAS directly, efforts have been put into targeting the downstream effector pathways, particularly the Raf-MEK-ERK^{59–61} and PI3K pathways.^{44,45} Raf kinase inhibitors, such as sorafenib (**12**)^{62,63} and vemurafenib (**13**)⁶⁴ (Figure 8), have shown impressive antitumor activity. However, the use of Raf inhibitors in RAS mutant cancer cells has been shown, paradoxically, to promote Raf-MEK-ERK signaling, thus precluding their use.⁹ Furthermore, data suggest that RAS mutant tumors are insensitive to single-agent PI3K inhibitors.⁹ On the other hand, certain inhibitors of MEK, such as selumetinib (**14**)⁶⁵ and GDC-0973 (**15**)⁶⁶ (Figure 8), have shown antiproliferative efficacy in RAS mutant cancers.

Daouti et al. recently screened compounds in an IMAP kinase assay (Molecular Devices) with a cRaf/MEK/ERK cascade.⁶⁷ This unique assay detects phosphorylation by the binding of an FITC-labeled ERK substrate peptide to a proprietary binding reagent bead.⁶⁸ To

determine MEK specificity, secondary assays with Caliper LabChip mobility shift kinase kits were utilized with cRaf and MEK/ERK cascades independently.⁶⁷ These assays measure the conversion of a fluorescent peptide substrate to a phosphorylated product on a microfluidic chip by separating the nonphosphorylated substrate from the phosphorylated product via electrophoresis and detection via laser-induced fluorescence.⁶⁹ Of the compounds screened, a new class of potent and selective MEK1/2 inhibitors, represented by RO4927350 (**16**; Figure 9) was identified.⁶⁷ In a cRaf/MEK/ERK cascade assay, compound **16** potently inhibited MEK1 kinase activity with an IC₅₀ of 23 nM. Interestingly, it also showed consistent MEK inhibition with ATP concentrations ranging from 2 to 250 μ M, which suggests that **16** is a non-ATP-competitive inhibitor and induces or stabilizes a novel fold in the MEK kinase. Compound **16** showed activity in the cRaf/MEK/ERK and MEK/ERK assays (with K_i values of 53 and 184 nM, respectively) but was not active in the cRaf assay (K_i > 50 μ M) when the compound was tested in the Caliper cRaf assay. Ambit's proprietary kinase profiling platform was used to assess the effects of **16** (10 μ M) on 227 different kinases, and it was found to be inactive against all other kinases, including cRaf and bRaf. The antitumor activity of **16** was investigated in vivo in a LOX human melanoma xenograft model in nude mice, containing the most common activating bRaf mutation (V600E). When **16** was administered orally to LOX tumor-bearing mice twice daily with doses ranging from 12.5 to 200 mg/kg for 11 days, dose-dependent antitumor activity was observed and partial or complete tumor regression was elicited at doses above 50 mg/kg. This compound was determined to have a unique mechanism of action: inhibiting both MEK and ERK phosphorylation simultaneously.⁶⁷

Lead optimization to improve the potency and PK–PD profile of **16** resulted in RO5068760 (**17**; Figure 9),⁷⁰ which contains a phenyl group in place of the thiazole in **16**. In vitro, **17** demonstrates MEK1 kinase inhibitory activity with an IC₅₀ of 0.025 $\pm$ 0.012 μ M in a cRaf/MEK/ERK cascade assay, a 2-fold improvement in potency compared to **16**. In vivo characterization of MEK 1/2 inhibition (as measured by ERK phosphorylation) in LOX melanoma and HT-29 colorectal cancer models gave estimated EC₅₀ values in plasma of 1.36 and 3.35 μ M, respectively. Furthermore, to produce 90% of tumor inhibition in vivo, plasma concentrations of **17** needed to be 0.65 or 5.23 μ M for bRafV600E or mutant KRAS tumor models, respectively. These data were similar to the IC₉₅ values (0.64 or 4.1 μ M) determined for cellular growth inhibition in vitro. Compound **17** is currently in preclinical development.

Tumor cell lines that harbor oncogenic mutation in both RAS and PIK3CA have been shown to be resistant to MEK inhibition.⁹ Thus, dual combinations of cRaf-MEK-ERK and PI3K inhibitors may be needed to effectively treat RAS mutant cancers. Indeed, combined inhibition of MEK and PI3K signaling showed good antitumor activity in mice with mutant KRAS-driven lung tumors.⁷¹ For example, when NVP-BEZ235 (**18**; Figure 9),⁷² a potent dual inhibitor of PI3K and mTOR, was combined with the MEK inhibitor **14**; there was marked synergy in shrinking mutant KRAS lung cancers.⁷¹ Additionally, after 2 days of the combination treatment, there was notable down-regulation of PI3K and ERK.

Inhibiting the Post-Translational Processing of KRAS

As shown in Figure 1, RAS proteins undergo a four-stage post-translational lipid modification: (1) farnesylation by FTase, (2) proteolytic cleavage of the terminal AAX motif by the CAAX protease RCE1, (3) carboxymethylation of the terminal cysteine by ICMT1, and (4) palmitoylation of cysteine residues close to the C-terminus of the protein.^{4,6} As membrane localization is required for the signaling and transforming capabilities of KRAS,⁷³ any of these points represent viable targets for therapeutic intervention.

FTase is an essential enzyme that attaches a 15-carbon isoprenoid farnesyl group to KRAS and is required for its proper localization and activity. Farnesyltransferase inhibitors (FTIs)^{74,75} fall into two categories: the CAAX peptidomimetics, including FTI-276 (**19**),⁷⁶ FTI-277 (**20**),⁷⁷ L-744832 (**21**),⁷⁸ B956 (**22**),⁷⁹ and FTI-2153 (**23**)⁸⁰ (Figure 10), and the non-peptidomimetics, which include tipifarnib (**24**),⁸¹ lonafarnib (**25**),⁸² and BMS-214662 (**26**)⁸³ (Figure 10). A number of recent publications have described their preclinical efficacy.^{4,75,84,85} In the case of compound **26**; the mean IC₅₀ values for HRAS farnesylation and KRAS farnesylation were determined to be 1.3 and 8.4 nM, respectively. Intravenous administration at 75 mg/kg compound **26** for 24 h led to 90% tumor cell kill in HCT-116 xenograft models.⁸³ While compound **26** was able to reverse the HRAS-transformed phenotype in vivo, it was unable to do the same for the KRAS-transformed phenotype, likely because of the potential geranylation of KRAS as an alternative to farnesylation. In fact, in the presence of FTIs the geranylation of KRAS is able to rescue its processing.⁸⁶

Compound **24** has displayed clinical efficacy as a single agent in elderly patients with acute myeloid leukemia (AML),^{87–89} myeloproliferative disorders,^{90,91} high-risk myelodysplasia,^{92–94} and multiple myeloma.⁹⁵ It has also been investigated in combination with etoposide for the treatment of AML: in a phase I trial 21 of 84 patients (25%) achieved complete remission, with a median complete remission duration of 9.8 months.⁹⁶ Additionally, compound **24** was investigated in combination with gemcitabine for the treatment of advanced pancreatic cancer: while the combination gave a favorable toxicity profile, overall survival was not prolonged compared to gemcitabine as a single agent.⁹⁷ Compound **25** has not shown promising clinical activity as a monotherapy for solid tumors, as phase II monotherapy trials for advanced pancreatic,⁹⁸ advanced colorectal,⁹⁹ and head and neck cancers¹⁰⁰ indicated single-agent activity to be <10%.¹⁰¹ However, **25** has shown some single agent activity in advanced hematologic malignancies.¹⁰² Combination of **25** and gemcitabine in a phase II trial as a second line treatment for advanced urothelial cancer gave a good response rate,¹⁰³ but a randomized phase III trial will be needed to assess the contribution of **25**.¹⁰¹

The loss of RCE1 has been shown to cause a mislocalization of RAS proteins and to reduce RAS-induced transformation in cells.^{9,104} In an effort to find small-molecule inhibitors of RCE1, Manandhar et al. screened the National Cancer Institute Diversity Set compound library in an in vitro assay utilizing yeast Rce1p.¹⁰⁵ In this high-throughput assay, diluted yeast (SM3164) membranes overexpressing the CAAX protease Rce1p were incubated with the compounds prior to the addition of a quenched fluorogenic substrate based on the KRAS

C-terminal sequence. Results were determined by measuring the fluorescence every 30–60 s over a 60 min time-course. Of the almost 2000 compounds screened, 46 inhibitors were initially identified in a RAS-based assay and 9 compounds (selected examples **27–30**; Figure 11)¹⁰⁵ were effective in a secondary assay with a yeast a-factor mating pheromone substrate peptide. The IC₅₀ values of these nine compounds were in the low micromolar range for both yeast (6–35 μ M) and human Rce1p (0.4–46 μ M). Additionally, copper complex **30** (Figure 11) showed good selectivity for Rce1p compared to the competitive CAAX protease Ste24p.

Disruption of ICMT1 results in a significant mislocalization of RAS proteins and has been shown to inhibit cell growth and KRAS-induced oncogenic transformation in nude mice.^{9,27} Using a radioactive HTS, Winter-Vann et al. discovered a small-molecule inhibitor of ICMT1.²⁷ The assay used measured ICMT1 activity by the incorporation of an [³H]methyl group into a farnesylated RCE1-proteolyzed KRAS substrate. In this assay, Sf9 insect cell membranes containing either recombinant RCE1 or ICMT1 were prepared and incubated with the library compounds. Results were assessed by the incorporation of a [³H]methyl group into a farnesylated, Rce1-proteolyzed KRAS substrate. Compounds displaying >50% inhibition of ICMT were screened in a secondary assay with an ICMT substrate biotin S-farnesyl-L-cysteine (BFC). The most potent compound was determined to be cysmethynil (**31**; Figure 12),²⁷ which gave an IC₅₀ of 2.4 μ M in the BFC assay. This compound was also shown to inhibit cell growth in an ICMT1-dependent fashion, cause mislocalization of RAS, and impair epidermal growth factor-induced MAPK signaling. Additionally, compound **31** displayed high specificity for ICMT, as it did not inhibit other enzymes in the prenylation pathway at 50 μ M including FTase, geranylgeranyltransferase type I, RCE1, an AdoMet-dependent DNA methyltransferase, and an unrelated protein methyltransferase.

As membrane localization is essential for KRAS activity, van der Hoeven et al.¹⁰⁶ developed a high-content assay to identify compounds capable of inhibiting KRAS membrane localization. The Prestwick chemical library was screened against wild-type Madin–Darby canine kidney epithelial (MDCK) cells or MDCK cells expressing green fluorescent protein (GFP) attached to the C-terminal 24 or 25 amino acids of KRAS (GFP-CTK) or HRAS (GFP-CTH). The cell lines were seeded in 96-well plates and exposed to the chemical library compounds for 48 h at 4 μ g/mL. Plates were imaged to assess the compounds' ability to displace the GFP-CTK or GFP-CTH from the plasma membrane. The hit identified was fendiline (**32**; Figure 13), which selectively and significantly mislocalized GFP-CTK compared to GFP-CTH. GFP-CTK was shown to be redistributed to endomembranes including the endoplasmic reticulum, Golgi apparatus, and cytosol. Furthermore, compound **32** inhibited downstream KRAS signaling and was able to block the proliferation of pancreatic, colon, lung, and endometrial cells. Compound **32** is a known L-type calcium channel blocker; however, as other classes of L-type calcium channel blockers were not shown to mislocalize KRAS, it is likely that the mechanism of disrupting KRAS membrane localization is not related to calcium channel blockade.

The farnesyl isoprenoid-containing small molecules salirasib (**33**) and TLN-4601 (**34**) (Figure 13) have also been shown to disrupt RAS membrane anchorage, leading to an inhibition of tumor cell growth.^{107–109} More specifically, these compounds are proposed to

act as antagonist of RAS function by competing for membrane-bound farnesyl-binding proteins located in the cell membrane.¹¹⁰ These compounds have been evaluated in a number of clinical trials with varying results.^{111–113}

EMERGING TARGETS AND NOVEL MECHANISMS

Many attempts to target mutant KRAS have been made; however, KRAS-related treatments have proved challenging in the clinic. Inhibitors of the upstream target epidermal growth factor receptor (EGFR), gefitinib (**35**) and erlotinib (**36**) (Figure 14), can significantly block KRAS signaling,¹¹⁴ but their effect is brief because of the rapid development of drug resistance. This often leads patients to develop a tumor recurrence that is generally refractory to chemotherapy and/or targeted therapy.

To elucidate the biological mechanisms that play a role in regulating aberrant KRAS signaling but that are independent from the KRAS signaling cascade, RNAi screening has been the technique of choice.¹¹⁵ RNAi research can be completed in a high-throughput manner and aims to identify a novel synthetically lethal interaction. Using this technique, Luo et al.¹¹⁵ found KRAS mutant cells to be hypersensitive to the inhibition of the mitotic kinase polo-like kinase 1 (PLK1).¹¹⁶ PLK1 plays an important role in the G2/M and M phases of the cell cycle and has been reported to have deregulated activity in various cancer cells.¹¹⁷ The PLK1 inhibitor BI-2536 (**37**; Figure 14)¹¹⁵ was shown to inhibit the proliferation of KRAS mutant DLD-1 cells at 25 nM by >50%. In contrast, wild-type KRAS DLD-1 cells were unaffected by the treatment. Compound **37** also showed a statistically significant reduction in tumor volume in a KRAS mutants DLD-1 xenograft model.

Luo et al. also showed that KRAS mutant cells are sensitive to proteasome inhibition.¹¹⁵ By use of the proteasome inhibitors MG132 (**38**) and bortezomib (**39**) (Figure 14), proteasome inhibition was shown to exhibit synthetic lethality in cells that harbor mutant KRAS.¹¹⁵ Additionally, researchers showed that paclitaxel (**40**; Figure 14)¹¹⁵ produced a specific and marked reduction in cell fitness for mutant KRAS cells. This indicates that cells harboring mutant KRAS are hypersensitive to mitotic disruption, as the mitotic cycle of wild-type KRAS cells was not statistically affected.

Kumar et al.¹¹⁸ identified the transcription factor GATA binding protein 2 (GATA2) as being essential for the survival of non-small-cell lung carcinomas (NSCLC) that harbor mutant KRAS. Indeed, deletion of GATA2 in KRAS mutant NSCLC xenografts models (via tamoxifen induction of GATA2^{Flox/Flox} mice) led to near-complete tumor regression. In another study investigating NSCLC, Puyol et al.¹¹⁹ identified cyclin-dependent kinase 4 (CDK4) as essential for propagating the survival of lung cells harboring mutant KRAS, as deletion of CDK4 led to immediate senescence and halted tumor progression in mouse models.

FUTURE PROSPECTS

HTS has been used to identify numerous compounds capable of disrupting the KRAS signaling pathway. However, no therapy capable of specifically and effectively targeting mutant KRAS is currently available. The problem is not due to a lack of therapeutic targets

but rather due to a lack of a comprehensive understanding of the biology underlying the disease. Cell signaling is often depicted as a linear cascade, and this model is used to identify potential therapeutic targets. However, the linear depiction of cell signaling is an oversimplification. In reality, cell signaling is complex and dynamic, in which one protein interacts with many others in a nonlinear, circuit-like fashion. Cancer is not caused by just one genetic mutation or aberrant protein that drives the disease. Instead, multiple mutations existing together form and drive the progression of cancer. Thus, effective treatment will require the regulation of many targets to attain a sustained response. Therefore, a therapy capable of inhibiting multiple pathways/targets is likely the best option for treating mutant KRAS-driven cancers.

KRAS can activate both PI3K and RAF, which lie in different signaling cascades. If only one pathway is inhibited, the cell can circumvent this event by overexpressing the other pathway and thereby create a resistant phenotype. For example, treatment with just the multikinase inhibitor PP-121 (**41**; Figure 15),¹²⁰ which is active against PI3K without blocking the RAS/RAF pathway, will likely be ineffective in patients. A better treatment option would be blocking both the PI3K and the RAS/RAF pathways, which could be achieved by the combination of a MEK inhibitor, such as **14**; with an AKT inhibitor, such as MK-2206 (**42**; Figure 15).¹²⁰ Inhibition of both pathways simultaneously may be able to produce better efficacy in mutant KRAS-driven cancers, as opposed to the inhibition of only one pathway.

The design of a successful cancer therapeutic relies on an understanding of the biology underlying the disease. The idea that cells signal in a linear fashion is quickly becoming antiquated and is being replaced with circuit-type models. Likewise, cancer treatments need to be designed under the notion of circuit signaling. We expect that employing this rationale will lead to better treatments, more effective drug combinations, and better outcomes for patients.

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ABBREVIATIONS USED

RAS	rat sarcoma
GTP	guanosine triphosphate
GDP	guanosine diphosphate
PPI	protein–protein interaction
GEF	guanine nucleotide exchange factor
GAP	guanosine triphosphatase activating protein
FTase	farnesyltransferase
RCE1	RAS-converting enzyme 1

ICMT1	isoprenylcysteine carboxylmethyltransferase 1
PTase	palmitoyl transferase
SH2	sarcoma homology 2
GRB2	growth factor receptor bound 2
RAF	rapidly accelerated fibrosarcoma
MAP	mitogen-activated protein
MEK	also known as MAPK, mitogen-activated protein kinase
ERK	extracellular signal-regulated kinase
ELK1	E twenty-six (ETS)-like transcription factor 1
PI3K	phosphoinositide 3-kinase
AKT	protein kinase B
NF-κB	nuclear factor κ light-chain enhancer of activated B cells
RalGDS	Ral guanine nucleotide dissociation stimulator
FOX	Forkhead box
PLC	phospholipase C
HTS	high-throughput screening
SC	sulfinyl cytidine
TPT	triphenyl
hTERT	human telomerase reverse transcriptase
ip	intraperitoneal
LT	large T
ST	small T
HPV	human papillomavirus
BJ	Bence Jones protein
RB	retinoblastoma
ROS	reactive oxygen species
MEF	mouse embryonic fibroblast
CTG	chemotaxis
BrdU	bromodeoxyuridine
SOS	son of sevenless
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect

IMAP	immobilized metal ion affinity based fluorescence polarization
FITC	fluorescein isothiocyanate
LOX	lipoxygenase
PK	pharmacokinetics
PD	pharmacodynamics
PIK3CA	phosphoinositide 3-kinase, catalytic α polypeptide
mTOR	mammalian target of rapamycin
FTI	farnesyltransferase inhibitor
BFC	biotin <i>S</i> -farnesyl-L-cysteine
AdoMet	<i>S</i> -adenosylmethionine
RNAi	RNA interference
PLK1	polo-like kinase 1
MDCK	Madin–Darby canine kidney epithelial
EGFR	epidermal growth factor receptor
NSCLC	non-small-cell lung cancer
CDK4	cyclin-dependent kinase 4
RTK	receptor tyrosine kinase

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drug inhibitor was discovered and is currently ready for preclinical development and further IND filing.

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Figure 1.

Post-translational processing of RAS proteins. Farnesyltransferase (FTase) catalyzes the transfer of a farnesyl group to the terminal cysteine of new synthesized RAS proteins. Next, the three C-terminal amino acids are cleaved by the endopeptidase RAS-converting enzyme 1 (RCE1). Carboxymethylation of the terminal cysteine residue is accomplished by isoprenylcysteine carboxymethyltransferase 1 (ICMT1). Finally, palmitoyl transferase (PTase) transfers a palmitoyl group to C-terminal cysteine residues of HRAS, NRAS, and the KRAS-4A isoform.

Figure 2.

Upstream signaling of RAS. Activated growth-factor-receptor tyrosine kinases undergo autophosphorylation and interact with the adaptor proteins SHC and GRB2. These proteins associate with various GEFs which exchange GDP for GTP, thereby activating the RAS protein. GAPs stimulate the RAS protein's intrinsic GTPase activity, causing the hydrolysis of GTP to GDP, thereby inactivating the protein.

Figure 3.

Downstream signaling of RAS. Ras interacts with a number of downstream effectors. The main ones include RAF protein kinases, phosphoinositide 3-kinases (PI3Ks), guanine nucleotide exchange factors for the RAS-related protein Ral (RalGDS), and phospholipase C ϵ (PLC ϵ).

Figure 4.
Structures of **1**; **2**; **3**; and **4**.

5 (Oncrasin-1)

Figure 5.
Structure of **5**.

Figure 6.
Structures of **6** and **7**.

Figure 7.

(a) Structures of compounds shown to interfere with the KRAS/SOS interaction. (b) Overlay of **8** (pink) and a compound similar to **11** (teal) in the hydrophobic binding pocket of KRAS. Reproduced with permission of D. Erlanson.⁵⁸

12 (Sorafenib) **13** (Vemurafenib) **14** (Selumetinib) **15** (GDC-0973)

Figure 8.
Structures of **12**; **13**; **14**; and **15**.

Figure 9.
Structures of **16**; **17**; and **18**.

Figure 10.
Structures of selected farnesyltransferase inhibitors.

Figure 11.
Structures of RCE1 inhibitors.

Figure 12.
Structure of **31**.

Figure 13.
Structures of **32**; **33**; and **34**.

Figure 14.
Structures that are active in the KRAS mutant synthetic lethal assays.

Figure 15.
Structures of **41** and **42**.