Molecular Therapy
Review

RAS degraders: The new frontier for RAS-driven cancers

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The function and significance of RAS proteins in cancer have been widely studied for decades. In 2013, the National Cancer Institute established the RAS Initiative to explore innovative approaches for attacking the proteins encoded by mutant forms of *RAS* genes and to create effective therapies for *RAS*-driven cancers. This initiative spurred researchers to develop novel approaches and to discover small molecules targeting this protein that was at one time termed "undruggable." More recently, advanced efforts in RAS degraders including PROTACs, linker-based degraders, and direct proteolysis degraders have been explored as novel strategies to target RAS for cancer treatment. These RAS degraders present new opportunities for RAS therapies and may prove fruitful in understanding basic cell biology. Novel delivery strategies will further enhance the efficacy of these therapeutics. In this review, we summarize recent efforts to develop RAS degraders, including PROTACs and E3 adaptor and ligase fusions as cancer therapies. This review also details the direct RAS protease degrader, RAS/RAP1-specific endopeptidase that directly and specifically cleaves RAS.

INTRODUCTION

The *RAS* family of genes including Kirsten rat sarcoma viral oncogene homolog (*KRAS*), neuroblastoma rat sarcoma viral oncogene homolog (*NRAS*), and Harvey rat sarcoma viral oncogene homolog (*HRAS*) encodes four proteins: KRAS4A, KRAS4B, NRAS, and HRAS.[1] The RAS proteins exhibit 82%–90% overall amino acid sequence identity with variations in the C-terminal hypervariable region and allosteric lobe.[1,2] The biological functions of the RAS isoforms vary based on membrane localization and effector use, which is controlled in part by post-translational modifications, including farnesylation, prenylation, methylation, and palmitoylation.[3] RAS proteins are guanosine triphosphatases (GTPases) with GTP occupancy regulated by cell-surface receptors, which control the activity of guanine nucleotide exchange factors to activate RAS and GTPase activating proteins that stimulate GTP hydrolysis to inactivate RAS. In the active state, RAS GTPases stimulate cell proliferation and survival through the control of cytoplasmic signaling cascades.[1,4] In addition, RAS is implicated in cell motility, polarity and morphology, differentiation, synaptic transmission, cell-cycle arrest and senescence, cytoskeletal rearrangements and pinocytosis, cytokinesis, and chemotaxis.[5] Considering the important role RAS proteins play in cell growth and survival, constitutively activating mutations to these genes can drive oncogenesis.[4]

Between 20% and 30% of all human cancers exhibit mutations in the *RAS* genes. This makes them some of the most frequently mutated genes in cancer.[6] Tumor cells express all three genes and mutations in any one gene can cause malignant transformation in cells and animal models.[7] Historically, *HRAS* has been the most studied of the *RAS* genes,[7] although *KRAS* is the most frequently mutated isoform (85%), followed by *NRAS* (11%) and *HRAS* (4%).[6] Of the missense gain-of-function mutations in all three *RAS* genes, 98% occur at one of three mutational hotspots: glycine-12, glycine-13, or glutamine-61, although 130 different missense mutations have been identified in tumors.[1,8] Each of these mutations produces distinct structural and biochemical effects on cellular signaling, leading to different clinical outcomes.[1] *RAS* mutations are considered cancer drivers, but remain important in long-term tumor survival,[9] making RAS an attractive therapeutic target for all cancers, including those with wild-type *RAS*.

Despite the high prevalence of *RAS* mutations in cancers, targeting RAS therapeutically has proven difficult. RAS has previously been termed "undruggable" because it lacks well defined binding sites and high concentrations of cellular GTP outcompete small molecules at its GTP-binding site.[4,10] The existence of four RAS proteins presents another challenge; these RAS isoforms are differentially expressed depending on the cell type, and cell lines vary considerably in how dependent they are on RAS signaling for growth and survival. In addition, *KRAS* is transcribed as two splice variants *KRAS4A* and *KRAS4B*, which produce proteins with distinct biological functions and post-translational modifications.[7] Researchers, however, have not been deterred in developing therapies against this elusive target. Here, we summarize the current landscape of RAS therapeutics, with an emphasis on the growing field of RAS degraders.

SMALL MOLECULE INHIBITORS OF RAS

Most therapeutic approaches have focused on the discovery of small molecules that target mutant KRAS. Here, we briefly explore the different inhibitors that have progressed into clinical and preclinical studies, as these have been extensively reviewed elsewhere.[11–22]

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Table 1 summarizes the state of selected KRAS small molecule inhibitors.

Mutant KRAS-specific small molecule inhibitors function by binding to the outer surface of KRAS in the expanded pocket of the switch II region.[4,73] These include covalent inhibitors that specifically bind only the G12C mutant KRAS protein found in 14% of non-small cell lung cancers (NSCLC), 5% of colorectal cancers, and 2% of pancreatic cancers.[74] The two most advanced small molecules in this class are AMG510 (sotorasib) and MRTX849 (adagrasib). In May 2021, the U.S. Food and Drug Administration (FDA) approved sotorasib for adult patients with KRAS G12C mutant locally advanced or metastatic NSCLC.[23,24,75] Both a phase III multicenter, randomized, open label, active-controlled study (NCT04303780) and expanded access protocol (NCT04667234) are currently underway.[23–26,75–78] For MRTX849, results have just been released on the KRYSTAL-1 study showing a 43% objective response rate in patients with KRAS G12C NSCLC. MRTX849 has now received a Breakthrough Therapy Designation status for previously treated patients with KRAS G12C NSCLC and is currently being reviewed by the FDA for accelerated approval. Many other KRAS G12C inhibitors are currently undergoing clinical trials (see Table 1).[49,79,80] Of note, two covalent G12C inhibitors, JNJ-74699157 and LY34994446, have recently failed clinical trials because of off-target toxicities.[23,71,72,81,82]

Progress has also been made targeting the G12D mutant of KRAS, which is present in 51% of pancreatic, 40% of colorectal, and 17% of lung adenocarcinomas.[22,83,84] These KRAS G12D inhibitors are currently all in preclinical development and include MRTX1133, BIKRASG12D1–3, KRA-533, and TH-Z835 (Table 1 and Figure 1). Notably, HRS-4642, a small molecule that targets G12D KRAS, is now entering phase I clinical trials (NCT05533463),[54,55] although supporting pre-clinical data are not publically available.

Other strategies have been explored for targeting multiple KRAS mutants locked in the "on" state. Revolution Medicines is developing multiple RAS-targeting molecules. One of these molecules, RMC-6236, is currently undergoing a phase I clinical evaluation for the treatment of patients with KRAS G12A, G12D, G12R, G12S, or G12V mutations. RMC-6236 is a RAS-MULTI(ON) inhibitor, selective for the active RAS(ON) form of both wild-type and mutant variants of the canonical RAS isoforms (HRAS, NRAS, and KRAS) (NCT05379985).[55,61,62]

Finally, approaches to inhibit RAS signaling more broadly include targeting indirect mechanisms,[85] such as preventing interaction of RAS with its effectors,[81,86] including the serine/threonine-protein kinases BRAF[65] and RAF1,[21,81,87–90] cyclophilin A,[91] and guanine nucleotide exchange factor SOS1.[1,23,56,70] The importance of RAS binding to the lipid membrane[23] and appropriate membrane localization for RAS signaling have also been targeted therapeutically by inhibiting the delta subunit of cGMP phosphodiesterase[92,93] or inhibiting palmitoylation (Figure 1).[94,95]

Overall, there has been substantial success with the discovery and preclinical and clinical development of RAS-directed small molecule therapeutics, which has culminated in improved patient outcomes. However, limitations to these therapies are important to consider. KRAS-independent tumors develop receptor tyrosine kinase dependency and signaling rebound kinetics[84] of downstream proteins including ribosomal S6 kinase, mammalian target of rapamycin, rapidly accelerated fibrosarcoma kinases (RAF), YES-associated protein 1, and phosphatidylinositol 3-kinase.[1,7,96,97] Alteration of these proteins and reactivation of RAS/MAPK signaling can result in outgrowth of resistant tumors.[84] Tumor resistance to KRAS inhibitors can also arise from mutations that result in loss of the tumor suppressor protein neurofibromin 1 or activation of another RAS isoform.[23,98–100] Resistance may also occur by promoting GDP and GTP exchange of RAS and indirect effects on the tumor microenvironment.[101–104] Another caveat is that some of these compounds do not readily enter cells, inhibiting their therapeutic potential. Finally, the current dominant approach of targeting the somewhat rare G12C- and G12D-mutant KRAS limits the number of cancers treatable by targeting RAS.[74,83] Indeed, 70% of cancers have a non-modified RAS or are heterozygous. Thus, there is a need for alternatives to small molecule targeting of mutant KRAS to expand the number of RAS-driven cancers that can be clinically treated, regardless of the association of RAS with GTP or GDP or its mutant status.

RAS DEGRADERS

Targeted protein degradation (TPD) has become a major focus of research over the past 10 years. Publications including PROteolysis Targeting Chimeras (PROTACs) or linker-based degraders, also known as bioPROTACs,[105] have gone from the single digits before 2015 to 450 publications in 2022.[106] Because only 20%–25% of all protein targets are being pursued in drug discovery efforts, TPD has focused on undruggable targets with degraders of >42 distinct protein targets published.[106] Unlike small molecules that bind to proteins to inhibit protein activity, degraders specifically target a protein inside cells for proteolysis using the physiologically normal process of disposing of old or damaged proteins, thus permanently removing the protein from the cellular pool. The targeted protein is first bound by the degrader molecule or protein and the bound molecule then recruits the cellular ubiquitination machinery to tag the protein for degradation by the 26S proteasome and recycling of its amino acids. Ubiquitination is performed by three enzymes, E1 (an activating enzyme), E2 (a conjugating enzyme), and E3 (a protein ligase).[107] Some degraders forego the linker approach to the ubiquitination pathway and instead directly cleave the target to become inactive and ultimately removed by normal cellular protein turnover (Figure 2).

To provide an alternative to targeting mutant RAS with small molecules, the recent development of RAS degraders has become a highly active and novel, therapeutic approach. As RAS is central in controlling cell proliferation, removal of RAS could be an effective strategy for the treatment of RAS-driven cancers. Degraders are in development that can treat all RAS isoforms (termed pan-RAS), as well as

Table 1. Summary of small molecules targeting KRAS

Selected small molecules targeting mutant KRAS							
Compound	Method of delivery	RAS Target	Concentration	*In vitro* studies	*In vivo* studies	Clinical trials	Ref(s).
AMG510 (Sotorasib or LUMA-KRAS)	Small molecule, His95 groove binder	KRAS G12C	0.010–0.123 μM in 22 cell lines that had heterozygous or homozygous *KRAS^{G12C}* expression	in *KRAS^{G12C}* cell lines, NCI-H358 and MIA PaCa-2, AMG 510 almost completely inhibited p-ERK (IC_{50} ≈ 0.03 μM) after a 2-h treatment	200 mg/kg AMG 510 resulted in inhibition of p-ERK in MIA PaCa-2 T2 and NCI-H358 tumors	Anticancer activity in 129 patients with locally advanced KRAS G12C-mutant solid tumors, Fast Track designation and undergoing a phase III study	23–26
MRTX849 (Adagrasib)	Small molecule, covalent KRAS G12C inhibitor	KRAS G12C	inhibited cell growth of KRAS^{G12C}-mutant cell lines with IC_{50} values ranging between 0.2 and 1,042 nM in the 3-D format	inhibited pERK; Thr^{202}/Tyr^{204} ERK1, pS6; Ser$^{235/236}$, and DUSP6, around 4.7 nM IC50s in KRAS^{G12C}-mutant H358 lung and MIA PaCa-2	Tumor regression in 17 of 26 (65%) KRAS^{G12C}-positive cell line- and patient-derived xenograft models from multiple tumor types at a maximum tolerable dose between 30 and 100 mg/kg/day	ORR was 43%, the DCR was 80% in 112 KRAS G12C NSCLC patients, received Breakthrough Therapy designation, undergoing phase III study	27–30
RG6330/GDC-6036	Small molecule, selective KRAS G12C inhibitor	KRAS G12C	IC_{50} of <0.01 μM	EC_{50} of 2 nM in K-RAS G12C-alkylation HCC1171 cells	Tumor growth inhibition in multiple KRAS G12C-positive cell lines and in xenograft mouse models	Recruiting patients for a phase Ia/Ib dose-escalation and dose-expansion study	31–33
D-1553	Small molecule, selective KRAS G12C inhibitor	KRAS G12C	ND	anti-tumor activity across a panel of cancer cell lines including lung, pancreatic and colorectal cancers with KRAS-G12C mutation	Highly potent *in vivo* in various cell line-derived xenograft tumor models with KRAS-G12C mutation	Undergoing phase I/II open label study	34,35
BI 1829311	KRAS^{G12C} selective small molecule inhibitor	KRAS G12C	ND	In a KRAS^{G12C} NSCLC cell line panel, downregulates DUSP6 and CCND1, and p-ERK	Daily oral dose of 60 mg/kg in a panel of lung and colon, mouse models showed comparable efficacy to AMG 510 and MRTX849	Recruiting patients for phase Ia/Ib, open-label, multicenter dose-escalation, and expansion study	36,37
JAB-21822	Covalent KRAS G12C inhibitor	KRAS G12C	<10–5000 nM in Ba/F3 cell lines bearing G12C mutation or secondary mutations	Cell growth inhibition in a variety of G12C mutant cancer cell lines	50-100% tumor growth inhibition in CDX (10 mg/kg PO daily) or PDX (100 mg/kg PO daily) mouse models bearing G12C mutations	Undergoing phase I/II study, in the 800 mg daily cohort, ORR = 50% and DCR = 100% with 4 non-confirmed PR	38–40
JDQ443	Small molecule covalent KRAS G12C inhibitor (GDP-bound)	KRAS G12C	0.02 μM in *KRAS G12C*-mutated NCI-H358 cells,	Currently optimizing compound potency, reduced cell proliferation and cRAF recruitment in NCI-H2122/NCI-H1437 and Ba/F3 *KRAS* mutants	30–100 mg/kg reduced tumor growth in multiple tumor xenograft and CDX models	Undergoing multiple clinical studies in G12C mutant NSCLC, CRC, and other patients	41–46
MK-1084	KRAS G12C inhibitor	KRAS G12C	ND	ND	ND	Phase I study alone and in combination with pembrolizumab in NSCLC KRAS G12C patients	47

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Table 1. *Continued*

Selected small molecules targeting mutant KRAS							
Compound	Method of delivery	RAS Target	Concentration	*In vitro* studies	*In vivo* studies	Clinical trials	Ref(s).
K20	inhibitor of KRAS G12C (GTP bound)	KRAS G12C	IC_{50} of 0.78 μM in H358 cells and 1.55 μM H23 mutant G12C cells	2.5 μM inhibited colony formation, induced apoptosis and reduced p-ERK levels in H23 and H358 cells	Tumor growth inhibition of 41% at 35 mg/kg and reduced p-ERK levels	NA	[48]
ARS-1620	atropisomeric selective KRASG12C inhibitor (GDP-bound)	KRAS G12C	$IC_{50} < 0.3$ μM across a panel of cancer cell lines harboring either *KRAS p.G12C* (H358, MIA-PaCa2, and LU65)	in 3 G12C mutant cell lines, 150 nM reduced cell viability and p-ERK expression, also tested in 2D and 3D systems	Tumor growth inhibition at 200 mg/kg daily in MiaPaCa2 and PDX mutant G12C models	NA	[49]
RM-018	KRAS G12C "tricomplex" inhibitor (GTP bound)	KRAS G12C	ND	attenuated both RAS-MAPK signaling and cell viability in cancer cell lines bearing $KRAS^{G12C}$mutations	Dose-dependent tumor regression in the NCI-H358 $KRAS^{G12C}$NSCLC xenograft mouse model	NA	[50,51]
SML-8–73-1, SML-10-70-1	small molecule, GTP-competitive inhibitor of K-RAS	KRAS G12C	26.6–100 mM	attenuated Akt and Erk phosphorylation at a concentration of 100 mM, antiproliferative effects in A549, H23, and H358 cells	NA	NA	[52,53]
HRS-4642	Small molecule that targets KRAS G12D	KRAS G12D	ND	ND	ND	Phase I study to evaluate the safety, tolerability, and pharmacokinetics in patients with advanced solid tumors with KRAS G12D mutations	[54,55]
BI-2852	KRAS inhibitor for the switch I/II pocket	KRAS G12D	binds to $KRAS^{G12D}$with a KD of 740 nM	inhibits GTP-$KRAS^{G12D}$ binding to SOS1, CRAF, and PI3Kα with an IC_{50} of 490, 770, and 500 nM pERK modulation and antiproliferative effects in NCI-H358	NA	NA	[56–59]
MRTX1133	noncovalent KRAS G12D inhibitor for the switch II pocket	KRAS G12D	0.2 pM	inhibited ERK phosphorylation in the AGS cell line with an IC50 of 2 nM in a 2D viability assay, the IC50 of MRTX1133 was 6 nM against the same cell line, with 500-fold higher selectivity against MKN1 cells	Antitumor activity with 94% growth inhibition observed at 3 mg/kg twice daily (i.p.) and tumor regressions of −62% and −73% observed at 10 and 30 mg/kg twice daily, in KRASG12D mutant Panc 04.03 cell line	NA	[60]
RMC-6236	RAS-MULTI(ON) inhibitor	RAS mutants	ND	ND	ND	Phase I clinical evaluation for patients with KRAS G12A, G12D, G12R, G12S, or G12V mutations	[55,61,62]

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Table 1. *Continued*

Selected small molecules targeting mutant KRAS							
Compound	Method of delivery	RAS Target	Concentration	*In vitro* studies	*In vivo* studies	Clinical trials	Ref(s).
KRA-533	small molecule KRAS agonist, binds the GTP/GDP-binding pocket of KRAS	KRAS mutants	10 μM	10 μM induced cell death in mutant KRAS cell lines A549, H157, Calu-1, and H292	7.5, mg/kg/day of KRA-533 i.p. for 28 days suppressed tumor growth in a dose-dependent manner in A549 xenografts	NA	63
THZ835	small molecule, binds GDP and GTP-bound KRAS, disrupts the KRAS–CRAF interaction	KRAS G12D	low μM range	reduced pERK and pAKT levels, increased p21 and p27 levels and reduced CDK2/4/6 and cyclin D1 expression cells went through G1 cell-cycle arrest and apoptosis	Mouse xenograft models of pancreatic cancer exhibited reduced tumor growth but mice experienced weight loss, suggesting the potential of off-target effects	NA	64
KAL-21404358	small molecule allosteric ligand against P110 site	KRAS G12D	K_D of 100 μM	impaired the interaction of K-$RAS^{G12D}$with B-Raf and disrupted the RAF-MEK-ERK and PI3K-AKT signaling pathways	NA	NA	65
12VC1	monobody, noncovalent inhibitor	KRAS G12V and G12C	expressed intracellularly alone or fused to VHL	inhibits ERK activation and the proliferation of RAS-driven cancer cell lines	H23 cells expressing VHL-12VC1 were significantly smaller than control tumors	NA	66
NS1 (aHRAS)	monobody, inhibits RAS-mediated signaling through targeting the α4–α5 surface	KRAS and HRAS	expressed intracellularly alone or fused to VHL	inhibited growth factor signaling and oncogenic H-RAS- and K-RAS-mediated signaling and transformation	NA	NA	3,67
Compound 3144	multivalent small-molecule, pan-RAS inhibitor	KRAS HRAS NRAS	K_d of 4.7/17/6.6/3.7 μM for KRAS G12D/KRAS wt/ HRAS/NRAS, respectively	lethality in cells partially dependent on expression of RAS proteins	Displays anti-tumor activity in breast and pancreatic xenografts	NA	68,69
DCAI (dichloro-2-methyl-3-aminoethyl-indole)	competitive inhibitor that blocks the RAS-SOS^{cat} interaction	KRAS, KRAS mutant	15.8 ± 0.4 μM	blocks the recruitment of the cRaf RBD-CRD domain to the cytoplasmic membrane, effect on cell viability ND	NA	NA	70

Two G12C inhibitors, JNJ-74699157 and LY3499446, are not included as these compounds failed clinical trials.[71,72]
Revolution Medicines is also developing RMC-6291, a $KRAS^{G12C}$ inhibitor, RMC-9805, a $KRAS^{G12D}$ inhibitor and RMC-8839, a $KRAS^{G13C}$inhibitor.
Jacobio Pharma is developing small molecule inhibitors: JAB-23400 (KRAS (multi)) and JAB-22000 (KRAS G12D).
Bridge Bio is performing preclinical studies for multiple compounds including the KRAS inhibitor BBP-454 and PI3Ka:RAS breakers.
CRC, colorectal cancer; DCR, disease control rate; ND, not disclosed; NA, information is not available; ORR, objective response rate; PI3K, phosphatidylinositol 3-kinase.

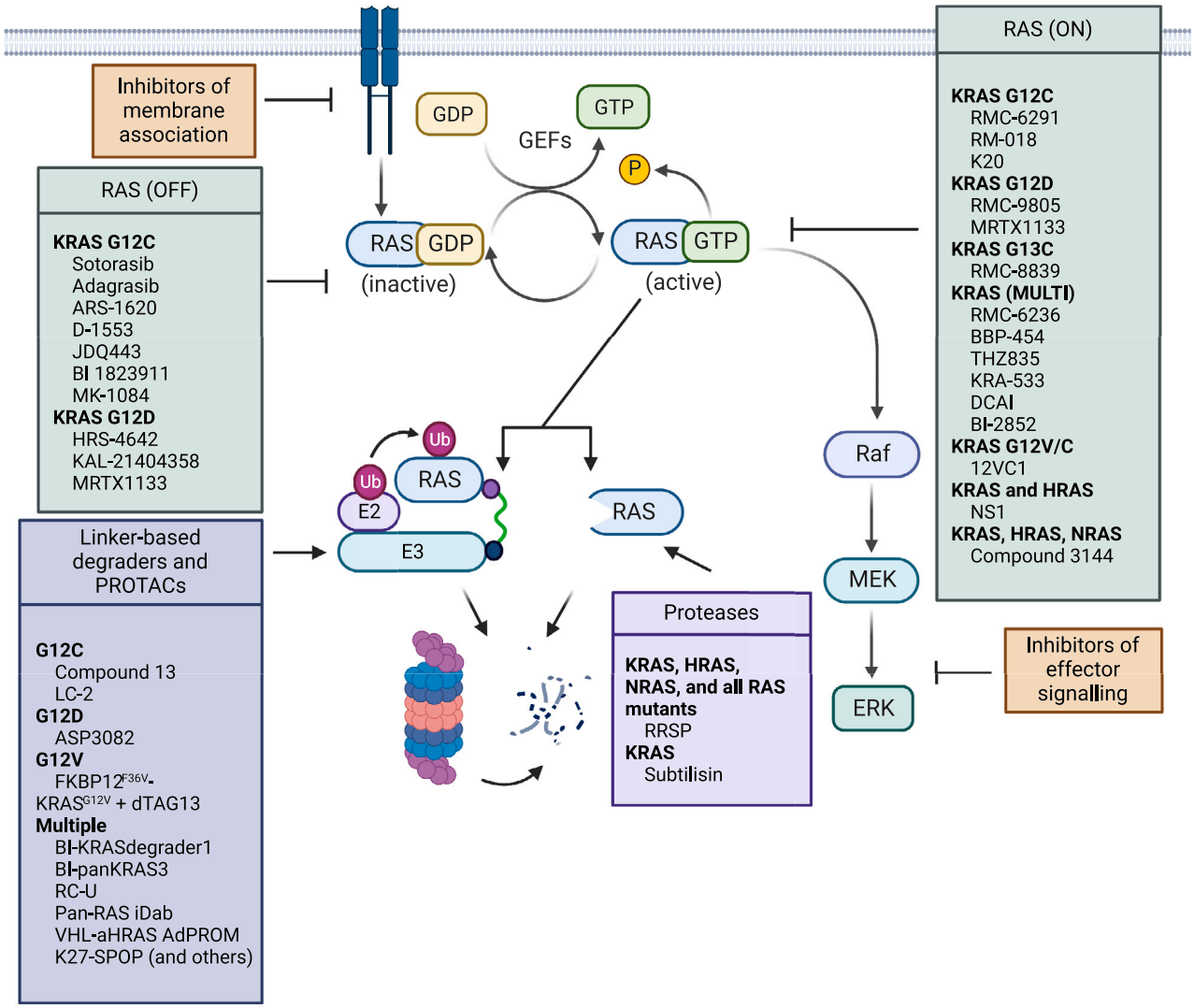

Figure 1. Schematic of selected RAS targeted therapeutics

Current RAS targeted therapeutics that are in pre-clinical and clinical development are grouped by mechanism of action. Only those that specifically target RAS are listed. See Table 1 for more details on *in vitro*, *in vivo*, and clinical studies. Figure created with Biorender.com.

degraders specific for RAS isoforms and even for mutant KRAS. Current early stage investigation, pre-clinical, and clinical trials are underway for multiple classes of degraders, including PROTACs, linker-based degraders, and direct proteolysis strategies.

PROTACs

PROTACs are novel, selective chemical ligands that target a protein of interest for degradation.[108] These molecules have one warhead with high specificity for the target with a second warhead that binds an E3 ligase adapter, thus bringing the protein of interest into close proximity of an E3 ligase substrate receptor protein, such as cereblon (CRBN). The targeted protein is ubiquitinated by E3 and thus redirected to the 26S proteasome for degradation.[10,109] The first instance of a PROTAC is when CRBN ligands were combined with an Src homology region 2 domain-containing phosphatase-2 (SHP2) inhibitor to promote SHP2 degradation.[15]

The first PROTAC targeting KRAS is compound 13 (also known as XY-4-88), which was created by fusing the KRAS G12C inhibitor ARS-1620 to CRBN. Although the compound permeates cells and recruits CRBN and KRAS G12C to a complex, the compound does not effectively degrade endogenous KRAS G12C.[109] The PROTAC approach has been successful when fusing the G12C inhibitor MRTX849 (adagrasib) with a chemical ligand that recruits the von Hippel-Lindau (VHL) E3 ligase complex. This molecule LC-2 sustains endogenous degradation of KRAS G12C and decreased ERK signaling when tested in KRAS G12C cell lines.[110] These compounds have yet to be tested *in vivo*.

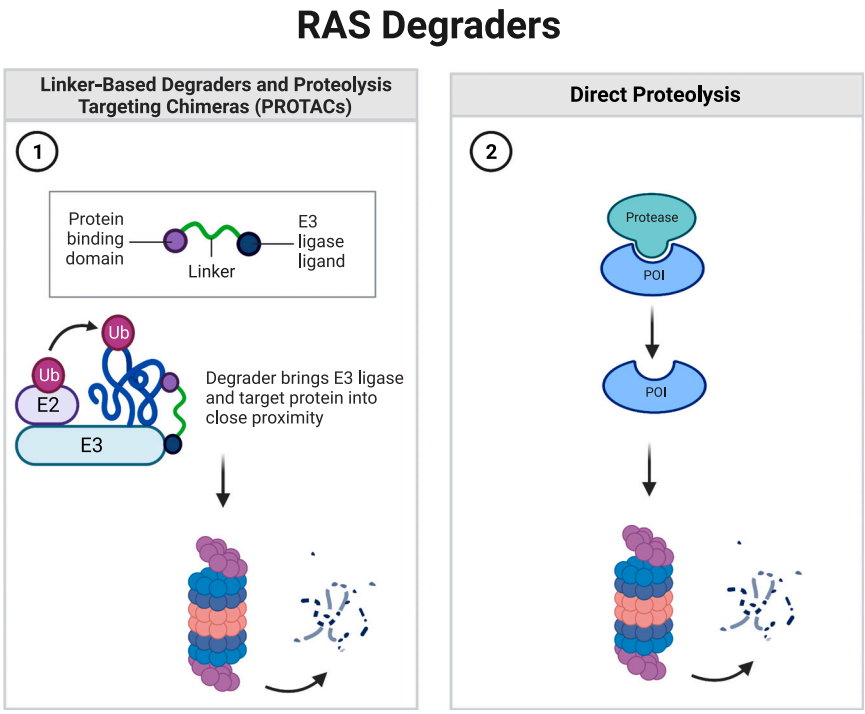

Figure 2. Schematic of RAS degrader mechanism of action

(1) Linker-based degraders and PROTACs fuse a protein binding domain or chemical compound to a ligand for an E3 ligase. The degrader creates a ternary complex between the protein of interest, such as RAS, and the E3 ubiquitin machinery.[10] E2 transfers ubiquitin to the protein of interest which allows it to be degraded by the 26S proteasome.[107] (2) Direct proteolysis occurs where the protease binds to and cleaves the protein of interest, for example, RAS, and the protein is subsequently degraded. Figure created with Biorender.com.

In data from the AACR 2021 conference, Boehringer Ingelheim reported a panKRAS degrader and a KRAS-specific PROTAC.[84,111] BIpanKRAS3 targets both KRAS and KRAS G12D. In a GP2d colon cancer cell line xenograft model, a 90 mg/kg dose of BIpanKRAS3 decreases pERK levels and nuclear protein Ki67 expression.[84,111] In addition, 30-mg/kg doses reduce tumor progression in the GP2d model and a KRAS G13D HCT15 cell line xenograft model. The BIKRASdegrader1 degrades mutant KRAS and inhibits pERK expression and cell proliferation in GP5d cells. This PROTAC degrades KRAS in a panel of KRAS mutants, but has not as yet been tested *in vivo*.

The most advanced PROTAC-like molecule in development is ASP3082, a selective KRAS G12D degrader. This molecule binds both KRAS G12D and an undisclosed E3 ligase adapter. An abstract from the 34th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics in October 2022 reports that ASP3082 degrades KRAS G12D in pancreatic cancer cells and exhibits dose-dependent reduction of pancreatic tumors after intravenous injection.[112] A phase I study of this molecule began in June 2022 for patients with previously treated locally advanced or metastatic solid tumors with the KRAS G12D mutation (NCT05382559).[113,114] See Table 2 for a summary of selected RAS PROTACs.

LINKER-BASED DEGRADERS

Linker-based degraders, also known as bioPROTACs,[105] are proteins rather than chemical ligands, but function similar to PROTACs. Linker-based degraders are generally plasmid-encoded fusion proteins that consist of a high-specificity target-binding protein domain linked to an E3 ubiquitin ligase or an E3 recruiting adapter. Upon transfection into cells, either the protein is ubiquitinated by the E3 ligase or the E3-recruiting adapter brings the corresponding E3/E2 complex to the vicinity of the target of interest and induces the transfer of polyubiquitin. In both cases, the targeted protein is tagged for proteasomal degradation (Figure 2).[74,106,126–128]

The first linker-based degrader that was specifically engineered to target RAS is the RC^{RAF-1}-U-Box (RC-U) degrader.[121] This expression construct expresses a fusion protein of the RAS binding (RBD) and cysteine-rich (CRD) domains of RAF1 with a U-Box family E3 ubiquitin ligase. Upon co-transfection, transient co-expression of a Flag-tagged mutant KRAS and RC-U reduces KRAS, HRAS, and NRAS protein levels and suppresses pancreatic cancer cell growth. For *in vivo* validation, PANC-1 xenograft tumors are injected with the expression plasmid in combination with *in vivo*-jetPEI (Polyplus transfection) reagent. When RC-U is transfected into tumors, RAS levels are reduced inside of the tumors and the rate of tumor growth is slowed.[121]

A distinct strategy to engineer degraders has taken advantage of the target specificity using antibody mimetics known as designed ankyrin repeats (DARPins). Expression plasmids have been generated to express the KRAS-specific DARPin K19 fused to the E3 adaptor ligand VHL under the control of a Tet-On promoter and H358 lung cancer cells have been stably transfected with these expression plasmids. In the presence of doxycycline to induce the promoter, the expressed KRAS degrader depletes only the KRAS isoform of RAS. Although the DARPin K19 does not specifically target mutant KRAS, cell proliferation is inhibited only in mutant KRAS cancer cells and the DARPin degrader has no effect on cells with wild-type KRAS or mutant NRAS or HRAS.[122] *In vivo* experiments have been performed by injecting mutant KRAS H358 cancer cells stably expressing the RAS degraders into CD-1 nude mice. Upon doxycycline treatment to induce expression of the degrader in the grafted tumor cells, the degrader directly decreases RAS levels within the tumor cells and tumor regression is observed.[122] Of note, no tumor decrease is observed in mouse xenografts of H1299 NRAS-mutant lung cancer cells,

Table 2. Summary of selected RAS degraders

Compound	RAS Targets	Concentration	*In vitro* studies	*In vivo* studies	Ref(s).
RRSP	KRAS, NRAS, HRAS	1-300 pM	Induces apoptosis or senescence in pancreatic, breast, and colon cancer	Tumor regression in pancreatic, breast, and colon xenografts	115–120
ASP3082	PROTAC against KRAS G12D	NA	ND	I*n vivo* results not disclosed but a phase I clinical trial began in June 2022	112–114
RC-U	KRAS, NRAS, HRAS	transfection	Reduced RAS levels upon transfection in HEK293T cells	Transfection reduced tumor size	121
VHL-KRAS DARPin K19	KRAS	transfection	Reduced KRAS levels in mutant KRAS cells	Doxycycline-inducible expression reduced tumor size	122
UBOX-Pan-RAS iDab	KRAS, NRAS, HRAS	transfection	Reduced RAS levels in mutant KRAS cells	Doxycycline-inducible expression reduced tumor size	122
BIpanKRAS3	KRAS, KRAS G12D	19–91 nM	Cleaved KRAS and reduced pERK levels in NCI-H2122 and SW837 cells	$KRAS^{G12/13D}$ mutant CDX xenograft models a 30-mg/kg dose caused tumor regression	84,111
Compound 13 (XY-4-88)	KRAS G12C	>0.410 μM	Did not degrade endogenous RAS	NA	109
LC-2	KRAS G12C	0.25–0.76 μM	Degraded endogenous KRASG12C and ERK	NA	110
$FKBP12^{F36V}$-$KRAS^{G12V}$ + dTAG13	KRAS G12V	transfection	Reduced KRAS G12V levels	NA	123
VHL-aHRAS AdPROM	KRAS, NRAS, HRAS	transfection	Reduced K,N,H-RAS in CRISPR-Cas9 HEK293 and U2OS cells	NA	124
DARPin K27-SPOP and others	various	plasmid and mRNA transfection	Several RAS degraders and E3 ligases reduced RAS expression and inhibited cell proliferation specific to each compound	NA	74
BIKRASdegrader1	KRAS mutants	2–116 nM	Degraded KRAS, reduced pERK levels and reduced cell proliferation	NA	111
Engineered subtilisin	KRAS	transfection	Cleaved eGFP-KRAS after doxycycline induction	NA	125

ND-not disclosed, NA-information is not available

validating the specificity of the DARPin for KRAS over other RAS isoforms.

In a parallel study, the KRAS-specific DARPin degrader has been directly compared with a pan-RAS inhibitor created by fusion of a pan-RAS intracellular single domain antibody (iDAb) with U-BOX E3 ubiquitin ligase. In contrast with the specificity of the DARPin for KRAS, the pan-RAS degrader based on the iDAb decreases protein levels of KRAS, HRAS, and NRAS and inhibits cell proliferation upon doxycycline induction across eight different tumor cell lines. The pan-RAS inhibitor decreases the growth of both the KRAS mutant H358 xenografts, as well as the NRAS-mutant H1299 xenografts.[122]

A similar bio-degrader has been developed using the affinity-directed protein missile (AdPROM) system target degradation. These AdPROMs are likewise created by the fusion of E3 ligases or adapters with peptidic binders with high affinity for proteins of interest.[124] To generate a RAS degrader, the engineered AdPROM has VHL fused to an HRAS/KRAS targeting monobody (aHRAS). When expression is induced in HEK293 and U2OS cells, the AdPROM decreases GFP-KRAS and H/N-RAS protein levels but has varying effects on cell proliferation, depending on the cell line.[3] It is notable that the effectiveness of the RAS-AdPROM to degrade RAS *in vivo* by demonstration of tumor reduction has not as yet been determined.

In a broad-based study to determine the efficiency of 10 different E3 adaptors, speckle-type POZ protein (SPOP) was identified as the most efficient to degrade GFP-KRAS when fused to an anti-GFP binding protein. SPOP has next been tested after fusion to six different RBD proteins, including those tested by other groups: RAF RBD, RAF RBD+CRD, the monobody aHRAS (referred to in this paper as NS1), and DARPin K19.[74] In addition, fusions have been created with DARPins K27 and K55, which have differential specificity for GDP and GTP-bound KRAS, respectively, and with R11.1.6, a RAS-specific combinatorial protein displayed in an SSo7d scaffold. All the fusions except for the DARPin K55 construct result in degradation of RAS when expressed in A549 lung cancer cells with the NS1 construct showing specificity for KRAS and HRAS, but not NRAS. This study suggests that RAS degraders can be tunable based on the ligand used to target RAS isoforms.[74] However, the effectiveness of these SPOP fusions to degrade RAS in tumors resulting in tumor reduction has not yet been demonstrated.

DIRECT PROTEOLYSIS DEGRADERS

Linker-based degraders and PROTACs are potent, versatile, and in theory could target any protein and be manufactured for tissue selectivity.[106,127,128] Technically, it is tedious to create these chemical and biological linkers. Challenges include the discovery of the optimal RAS targeted ligand and pairing it with the best linker to the ubiquitination machinery. The final and perhaps most straightforward method of inhibiting RAS is through direct proteolysis, eliminating the process of bringing together multiple proteins in a complex. Direct proteolysis works by introducing a proteolytic enzyme into the cell that can cleave a target protein with high specificity, thus inhibiting its function.

One instance of engineered direct proteolysis against RAS has been completed by modification of the *Bacillus* protease subtilisin. Subtilisin is a canonical serine protease that was re-engineered to specifically cleave the conserved QEEYSAM amino acid sequence, which is found in the switch II region of RAS. In human cells with fluorescently tagged RAS (eGFP-KRAS), engineered subtilisin can self-activate, locate eGFP-KRAS at the plasma membrane, and cleave it as indicated by the presence of the eGFP fusion product and the precipitous disappearance of KRAS.[125] In HEK293T cells, expression of the protease induced by doxycycline decreases eGFP-RAS expression.[125] The function of RAS targeted subtilisin on endogenous levels of RAS is currently unknown and the effectiveness of this protein to reduce tumors has not as yet been evaluated.

Perhaps the most unique approach to a RAS degrader takes advantage of the multiple bacteria that produce protein toxins that have evolved over thousands of years to specifically target RAS. These, along with other bacterial effectors, could be harnessed for use as therapies.[129,130] The most advanced and well studied RAS protease was first reported in 2014 and over the past 9 years has been developed into the most advanced proteinaceous RAS degrader in preclinical development.

The RAS/Rap1-specific endopeptidase (RRSP) is naturally produced by the bacterium *Vibrio vulnificus*. Originally termed domain of unknown function in the fifth position (DUF5), RRSP is now recognized as a highly potent cytotoxic effector domain from the multifunctional-autoprocessing repeats-in-toxin (MARTX) toxin.[131] The X-ray structure of RRSP reveals that it is a three-domain protein with a membrane targeted domain and a catalytic domain in the same protease family as the eukaryotic Wnt-specific protease TIKI (Figure 3).[132] RRSP as found in MARTX toxins has cytotoxic activity[115] and blocks RAS-MAPK signaling by cleaving RAS and the closely related repressor activator protein 1 (RAP1) within the switch I region between the residues tyrosine-32 and aspartate-33.[116] The enzyme is highly specific for the RAS/RAP1 switch I sequence, such that even the closely related RAS-like proto-oncogene A GTPase is not cleaved by RRSP. This activity is also found in other bacterial toxins, including *Photorhabdus spp*. insect pathogens and *Aeromonas spp.* fish pathogens.[115] RRSP cleaves HRAS, NRAS, and KRAS as well as KRAS G12V, G12D, G12C, G13D, and Q61R mutants and is highly potent, with complete degradation of RAS occurring in the picomolar range.[117,118] RRSP has activity against both GDP- and GTP-bound RAS, increasing its efficacy.[117,118] The cleavage of RAS results in downstream loss of phosphorylated ERK, but has varying effects on cell proliferation based on the cell line. Colon carcinoma HCT116 and SW1463 cell lines are highly susceptible to RRSP and undergo apoptosis, while RRSP treatment of GP5d and SW620 colon cells induces G1 cell-cycle arrest. In fact, the predominant result of cleaving all RAS in the cell is to initiate CDK2 cell-cycle arrest. RRSP has also been linked to CDK1 cell-cycle arrest and rescues expression of the tumor suppressor

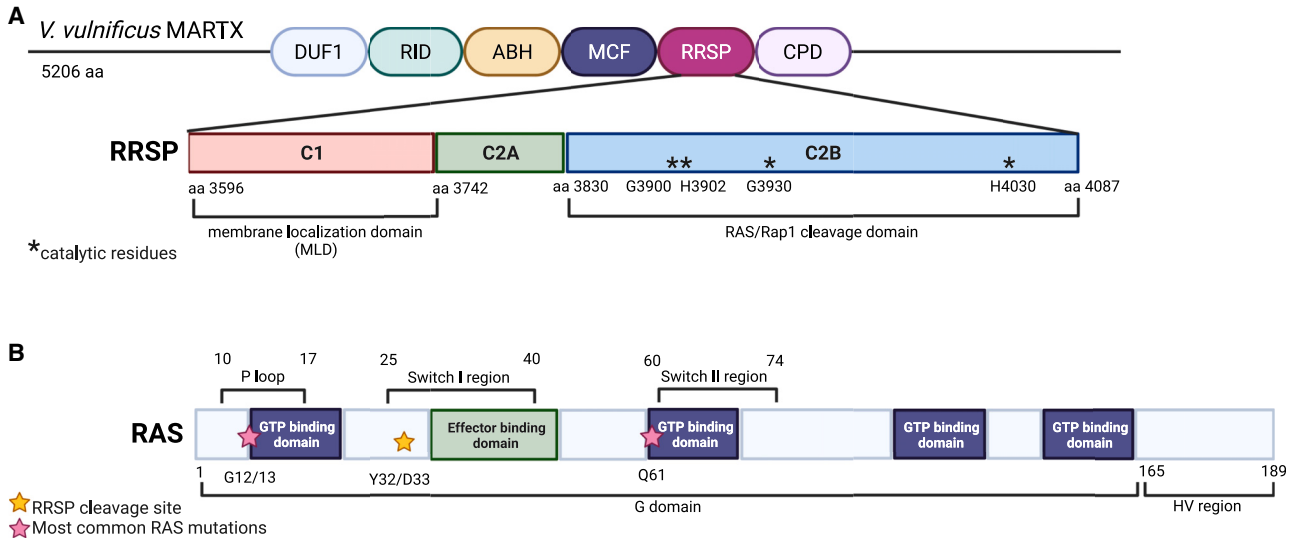

Figure 3. Functional domains within RRSP and RAS

(A) Schematic of RRSP from the *Vibrio vulnificus* MARTX toxin. The *V. vulnificus* MARTX is composed of six effector domains, including RRSP. The RRSP effector has three functional domains: C1, C2A, and C2B. C1 is the membrane localization domain and C2B contains the catalytic domain of RRSP. The function of the C2A domain is unknown. Within the C2B domain, four residues (denoted with asterisks) are critical for RAS/Rap1 cleavage. These include G3900, H3902, G3930, and H4030. If these residues are mutated catalytic activity is significantly reduced.[115,117,132] (B) Schematic of the key functional domains of the RAS proteins. The RAS proteins (HRAS, NRAS, KRAS4A, and KRAS4B) have high sequence homology other than the C-terminal domain termed the hypervariable (HV) region. RAS proteins contain a P loop and Switch I/II regions.[4,10] These three domains all are subject to the most common mutations found in RAS-driven cancers. The P loop and Switch II region each contain a GTP-binding domain and are the sites for the most frequent mutations G12/13 and Q61 (denoted with pink stars). The Switch I region contains the effector binding domain. RRSP cleaves RAS within this region between the residues Y32 and D33 (denoted with a yellow star), thus interfering with RAS downstream effector activation. Figure created with Biorender.com.

p27.[118] Further, RRSP from *Photorhabdus luminescens* inhibits cell proliferation, disturbs mitotic progression via CDK1, and increases the rate of HeLa cell death.[133] Comprehensive analyses using both the NCI-60 cell line panel and a pancreatic cancer cell panel show that most tumor types are sensitive to RRSP, with those exhibiting RAS genomic abnormalities being the most sensitive to RRSP.[118–120]

To demonstrate the function of RRSP *in vivo*, RRSP has been engineered as a chimeric toxin with RRSP fused to the translocation B fragment of diphtheria toxin (RRSP-DT_B). DT_B binds heparin-binding epidermal growth factor-like growth factor (HB-EGF) which is at least 1,000-fold less potent in cells expressing murine HB-EGF than in cells expressing human HB-EGF, making this an important system for testing the *in vivo* efficacy of RRSP.[119] Intra-peritoneal administration of RRS-DT_B at 0.1 mg/kg decreases total RAS expression in xenograft tumors. This results in tumor growth inhibition for wild-type and mutant KRAS triple-negative breast and in wild-type and KRAS-mutant colorectal xenografts,[119] as well as a decrease in KRAS-mutant pancreatic patient-derived tumor xenografts.[120] This protein is also stable for up to 16 h in the bloodstream of immunocompetent mice. Challenges remain for how to best deliver RRSP only to tumors. Loftis et al.[134] have shown that RRSP fused to another toxin, anthrax toxin protective antigen, is specifically delivered to only cancer cells by targeting to epidermal growth factor receptor or carcinoembryonic antigen. In addition, RRSP-DT_B has been redirected by changing the toxin to engage the IL-2 receptor.[119] Thus, for the proteinaceous RAS degraders, the depth of data both *in vitro* and *in vivo* sets RRSP apart from other degraders as the most advanced in development.

ENHANCING THE DELIVERY OF RAS THERAPEUTICS

All of the RAS degrader strategies have the potential to have significant issues with delivery, as they require cytoplasmic exposure within the solid tumor for target engagement and efficacy. PROTACs have a high molecular weight because of the two-warhead design; in preclinical studies, some have demonstrated issues with membrane permeability, solubility, and pharmacokinetics.[135] The cells are not taking up these molecules spontaneously. Because of these issues, many of these PROTACS have yet to be tested *in vivo* and may not show *in vivo* efficacy with exogenous addition or systemic injection. The success of ASP3082 in mouse xenograft studies and the start of a phase I clinical trial suggests that at least one PROTAC molecule has been developed to surmount the delivery barrier. However, the difficulty in development of these molecules may decrease the overall success to generate molecules beyond ASP3082 that can target pan-RAS or other mutant RAS proteins.

The delivery barrier is more profound for both the linker-based and directed proteolysis degraders, as these large biological proteins need to be translocated within the cells. The fusion RRSP-DT_B demonstrates this barrier can be surmounted by using toxin-based

cytosolic delivery, but other degraders in development have not yet been tested in tumor models. Further, although toxins can be used for the delivery of RRSP protein and potentially other protein degraders, these biologics must still be re-engineered for targeting against each type of cancer, which could ultimately limit clinical applications.

A new frontier in RAS therapeutics may be through targeting and delivery with mRNA. In the wake of the success of mRNA vaccines during the coronavirus disease 2019 (COVID-19) pandemic, these therapies have become attractive targets for cancer treatments. Emerging evidence suggests *KRAS* mutant cancers are attractive targets for immune-based treatments in pancreatic and other cancers. Advances to target KRAS-driven cancer with mRNA-encoded proteins are already significantly advanced. The mRNA vaccine, mRNA-5671, which encodes neoepitopes for common KRAS mutations (G12C, G12D, G12V, and G13D) is delivered via a lipid nanoparticle. A phase I clinical trial (NCT03948763) in patients with NSCLC, colorectal cancer, and pancreatic adenocarcinoma has closed enrollment in 2022.[84,136,137] RAS has also been targeted via AZD4785, a constrained ethyl-containing therapeutic antisense oligonucleotide. AZD4785 is complementary to a sequence in the 3′-UTR of *KRAS* mRNA. AZD4785 targets both the mutant and wild-type KRAS isoforms for ribonuclease H-mediated degradation without any delivery agent. *In vivo,* AZD4785 results in tumor growth inhibition in NCI-H358 KRAS mutant lung cancer xenografts, LXFA 983 patient-derived xenograft models, and several additional tumor models.[6] However, in a clinical trial (NCT031018390) AZD4785 did not reduce KRAS levels, although work is ongoing to enhance its efficacy.[23] In addition, a KRAS G12D mutant-selective small interfering RNA, siG12D LODER, has shown antitumor activity in mouse models of pancreatic cancer. In a phase I trial in combination with chemotherapy in 12 pancreatic cancer patients, 2 patients are shown to have a pathological response and 10 achieved stable disease (NCT01188785).[138] A phase II trial in patients with KRAS G12D pancreatic cancer is currently underway (NCT01676259).[23,139] KRAS G12D-directed small interfering RNA is currently being evaluated in pancreatic cancer[6] and a short hairpin RNA against c-RAF in KRAS mutant lung cancers has led to partial tumor regression.[140]

Given these successes, the opportunity to pair RAS degrader strategies with mRNA delivery seems a promising approach. The linker-based RAS degrader comprised of DARPin K27 paired with SPOP is successfully expressed in cancer cells from a transfected mRNA and expression is correlated with a decrease in RAS levels.[74] This is the first RAS degrader shown to work by a method beyond plasmid transfection or toxin-based protein delivery. The DARPin K27-SPOP mRNA, however, has not yet been tested for *in vivo* for tumor reduction.

As seen for BNT162b2, or mRNA-1273, to prevent COVID-19, the delivery of mRNA can be achieved through multiple types of nanoparticles.[141] A variety of nanoparticle types have been created with compositions that alter their delivery properties, as discussed elsewhere.[141–143] Most recently, an mRNA that expresses RRSP (referred to in the paper by the previous name DUF5) has been successfully delivered to colon cancer cells using a nanoparticle delivery system. The mRNA expressing RRSP degrades RAS in mutant HCT-116 (KRAS G13D) and H358 (KRAS G12C) cells. *In vivo,* RAS levels are decreased after the injection of RRSP mRNA-loaded nanoparticles in colorectal xenografts and lung adenocarcinoma, and the treatment inhibits tumor growth. The efficacy is comparable with the mutant KRAS inhibitor AMG510.[144] Thus, a strategy is likely underway for implementation with other developed RAS degraders for *in vivo* applications and therapeutic development.

IMPACT ON BASIC CELL BIOLOGY

The development of RAS degraders has the potential not only to expand therapeutic options, but also to expand our toolbox for studying RAS signaling pathways. *RAS*-less mouse embryonic fibroblasts[145] and RNAi technologies[146] are extensively used to study the impact of loss of *RAS* on cell biology and signaling. PROTACs and RRSP-DT_B can similarly render cells RAS-less and have the advantage of being exogenously added inhibitors that can be used in a dose-dependent fashion. Linker-based degraders under control of the TET-on promoters also provide dose-dependent control of RAS levels. Several studies have been conducted that take advantage of these degraders to understand cell signaling.

A final unique iteration of linker-based degraders to decrease RAS expression is seen by the fusion of KRAS G12V to the linker $FKBP12^{F36V}$ (dTAG). In cells expressing this fusion protein, RAS expression is decreased after the treatment of cells with the chemical molecule dTAG13, which binds both $FKBP12^{F36V}$ and the CRBN E3 ligase. dTAG13 brings the RAS complex into close proximity of the ubiquitin machinery and, thus, decreases KRAS G12V expression and cell proliferation in NIH 3T3 mouse cells. The approach in combination with proteomics and transcriptomic profiling has been used to study the impact of KRAS degradation on signaling. This approach could conceptually also be employed *in vivo* with xenografts that stably express $FKBP12^{F36V}$ targeted against RAS. As proof of concept, a luciferase fusion to $FKBP12^{F36V}$ is degraded *in vivo* after the injection of dTAG13, but a similar study of the impact of RAS degradation has not yet been developed.[123]

Similarly, the AdPROM system is also valuable for studies of the impact of RAS depletion on cell signaling. A ligand-inducible AdPROM (L-AdPROM) created by addition of the PROTAC to FLAG-Halo-aHRAS expressing A549 cells also degrades RAS levels.[147] Finally, as described above, RRSP-DT_B can render cells RAS-less, and these cells have been used in phospho-proteomic profiling to demonstrate that depletion of all RAS proteins induces cell-cycle arrest while cell death by apoptosis or senescence varies by cell line.[118] Thus, the development of RAS degraders could be excellent tools for many cell-based and *in vivo* basic science studies, even if the molecules are not ultimately forwarded for clinical development.

CONCLUSIONS

The field of RAS therapeutics has quickly expanded and presents great potential for future basic science and clinical applications. Specifically, the new field of RAS degraders alongside enhanced drug delivery technologies may benefit the 20%–30% of patients with RAS-driven cancers. Advancement of ASP3082 to clinical trials indicates that success is possible with chemical ligands being added to the small molecule repertoire of RAS-targeting therapeutics. Biologics are advancing through preclinical trials with the potential of tunable and also highly specific KRAS and pan-RAS degraders to be developed. In this class of protein biodegraders, RRSP is the only degrader that has shown *in vivo* efficacy across several different models, including breast, colon, and pancreatic mouse models, which is, thus far, the only degrader that reduced tumors by both exogenous protein addition and mRNA delivery. Indeed, investigating other bacterial proteases like RRSP presents untapped potential in terms of cancer therapeutics. RAS degraders present exciting potential to develop therapies that target not only KRAS mutants, but also wild-type RAS, which can expand our understanding of basic science and reach a broader population of cancer patients.

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AUTHOR CONTRIBUTIONS

T.E.E. conducted the investigation, wrote the original draft of the manuscript, and prepared the figures and tables. T.E.E. and K.J.F.S. revised the subsequent drafts and approved the final version of the manuscript.

DECLARATION OF INTERESTS

K.J.F.S. discloses that she holds a patent on use of RRSP as a cancer therapeutic (Patent # US10829752B2). K.J.F.S. has a significant interest in Situ Biosciences, a contract research organization that conducts research unrelated to this work.

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