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REVIEW ARTICLE

Covalent inhibitor targets KRas^{G12C}: A new paradigm for drugging the undruggable and challenges ahead

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Abstract *KRAS* is one of the most commonly mutated oncogenes in cancers and therapeutics directly targeting the KRas have been challenging. Among the different known mutants, KRas^{G12C} has been proved to be successfully targeted recently. Several covalent inhibitors selectively targeting KRas^{G12C} have shown promising efficacy against cancers harboring *KRAS*^{G12C} mutation in clinical trials and AMG510 (sotorasib) has been approved for the treatment of *KRAS*^{G12C}-mutated locally advanced or metastatic non-small cell lung cancer. However, the overall responsive rate of KRas^{G12C} inhibitors was around 50% in patients with non-small cell lung cancer and the efficacy in patients with colorectal cancer or appendiceal cancer appears to be less desirable. It is of great importance to discover biomarkers to distinguish patients who are likely benefitted. Moreover, adaptive resistance would occur inevitably with the persistent administration like other molecularly targeted therapies. Several combinatorial regimens have been studied in an effort to potentiate the efficacy of KRas^{G12C} inhibitors in preclinical settings. This review summarized the recent progress of covalent KRas^{G12C} inhibitors with a focus on identifying biomarkers to predict or monitor the efficacy and proposing rational drug combinations based on elucidation of the mechanisms of drug resistance.

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Abbreviations

AKT	protein kinase B
ALK	anaplastic lymphoma kinase
APC	adenomatous polyposis coli
AURKA	Aurora kinase A
CDK1	cyclin-dependent kinase 1
CDK4	cyclin-dependent kinase 4
CRC	colorectal cancer
CXCL8/10/11	C-X-C motif chemokine ligand 8/10/11
DCR	disease control rate
DUSP	dual-specificity phosphatases
EGFR	epidermal growth factor receptor
EMT	epithelial—mesenchymal transition
ERK	extracellular regulated protein kinases
FDA	U.S. Food and Drug Administration
FGFR	fibroblast growth factor receptor
GAPs	GTPase activating/accelerating proteins
GDP	guanosine diphosphate
GEFs	guanine nucleotide exchange factors
GI	gastrointestinal/gastro-intestinal
GM-CSF	granulocyte-macrophage colony-stimulating factor
HER2	human epidermal growth factor receptor-2
IC_{50}	half maximal inhibitory concentration
IFN-γ	inactivation of interferon-γ
IL-6/10	interleukin-6/10
KEAP1	Kelch-like ECH-associated protein 1
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LKB1	liver kinase B1
MAPK	mitogen-activated protein kinase
MET	hepatocyte growth factor receptor
MHC	major histocompatibility complex
MMP2	matrix metallopeptidase 2
NSCLC	non-small cell lung cancer
ORR	overall response rate
OS	overall survival
PD-1/PDL-1	programmed cell death protein-1/ programmed death ligand-1
PDX	patient-derived xenograft
PFS	progression-free survival
PI3K	phosphoinositide-3-kinase
Raf	Ras-associated factor
RAL-GEFs	Ral-specific guanine nucleotide exchange factors
RAS	rat sarcoma protein
RB	retinoblastoma
RET	proto-oncogene tyrosine-protein kinase receptor Ret
RNAi	RNA interference
RTK	receptor tyrosine kinase
SAR	structure activity relationships
SHP2	Src homology containing protein tyrosine phosphatase 2
SOS1	SOS Ras/Rac guanine nucleotide exchange factor 1
STK11	Serine—Threonine Kinase 11
TGFβ	transforming growth factor beta
TGI	tumor growth inhibition
TP53	tumor protein p53

Introduction

RAS gene was first discovered in 1964[1] as a viral gene transferred from the rodent genome and is part of an RNA sarcoma virus with potent oncogenic functions.[2] There are three *RAS* family members in human: *HRAS*, *NRAS* and *KRAS*. *KRAS* appears in forms of *KRAS4A* and *KRAS4B* as a result of alternative splicing,[3] while the latter one is the predominant form in human.[4] *KRAS* encodes a 21 kDa GTP-binding protein and known as Ras-like GTPase.[4] It switches between active and inactive state by binding to GTP or GDP respectively.[5] This process is regulated by guanine nucleotide exchange factors (GEFs) which facilitate the release of GDP, and GTPase activating/accelerating proteins (GAPs) that strengthen the relatively poor intrinsic GTPase activity of KRas.[3] In response to extracellular stimuli, KRas binds to GTP and becomes active after conformational switching,[6] which connects activated membrane receptors to mitogen-activated protein kinase (MAPK) pathway,[7] phosphoinositide-3-kinase (PI3K) pathway[8] and Ras-like small GTPases (Ral) pathway[4] (Fig. 1), thus playing important roles in regulating cell growth, survival, differentiation, proliferation and migration.[2] In consistency to its pivotal physiological roles, KRas is indispensable for the development of mice embryo and plays a key role in cardiovascular homeostasis.[9] However, knockout of *NRAS* or *HRAS* showed no effect on the viability of mice[10] and replacement of the *KRAS* with *HRAS* did not lead to embryonic lethality,[11] indicating the redundant while distinctive roles of *RAS* family members.

KRas is frequently hyper-activated in cancers as a result of gain-of-function mutation or abnormal activation of upstream receptors.[12] Although mutations occur in all three *RAS* members,[3] mutations in *KRAS* are the most frequent, which accounts for about 85% of *RAS* mutations in human cancers, especially in pancreatic cancer,[13] colorectal cancer[14] and lung cancer[4] (Fig. 2). *KRAS* mutations mainly occur at codon 12, 13 or 61.[4] Mutations at codon 12 are the most common, including G12C, G12V and G12D.[15] G12C mutation occurs in 15% of lung cancer and 8% of colorectal cancer. Mutation at G12 interferes the binding of Ras with GAP and inhibits GTP hydrolysis stimulated by GAP,[4] thus keeping Ras in the active GTP-bound form.[8] Mutated *KRAS* leads to the continued phosphorylation and activation of the extracellular signal-regulated kinase (ERK), which in turn phosphorylates multiple cytoplasmic proteins, cytoskeletal proteins, and transcription factors that initiate genetic programs associated with cell growth and survival.[16–18] Activated Ras has long been considered a prominent tumor driver.[19] Ras activation had been identified in various carcinogen-induced animal tumor models nearly four decades ago.[20–22] The roles of *RAS* in the

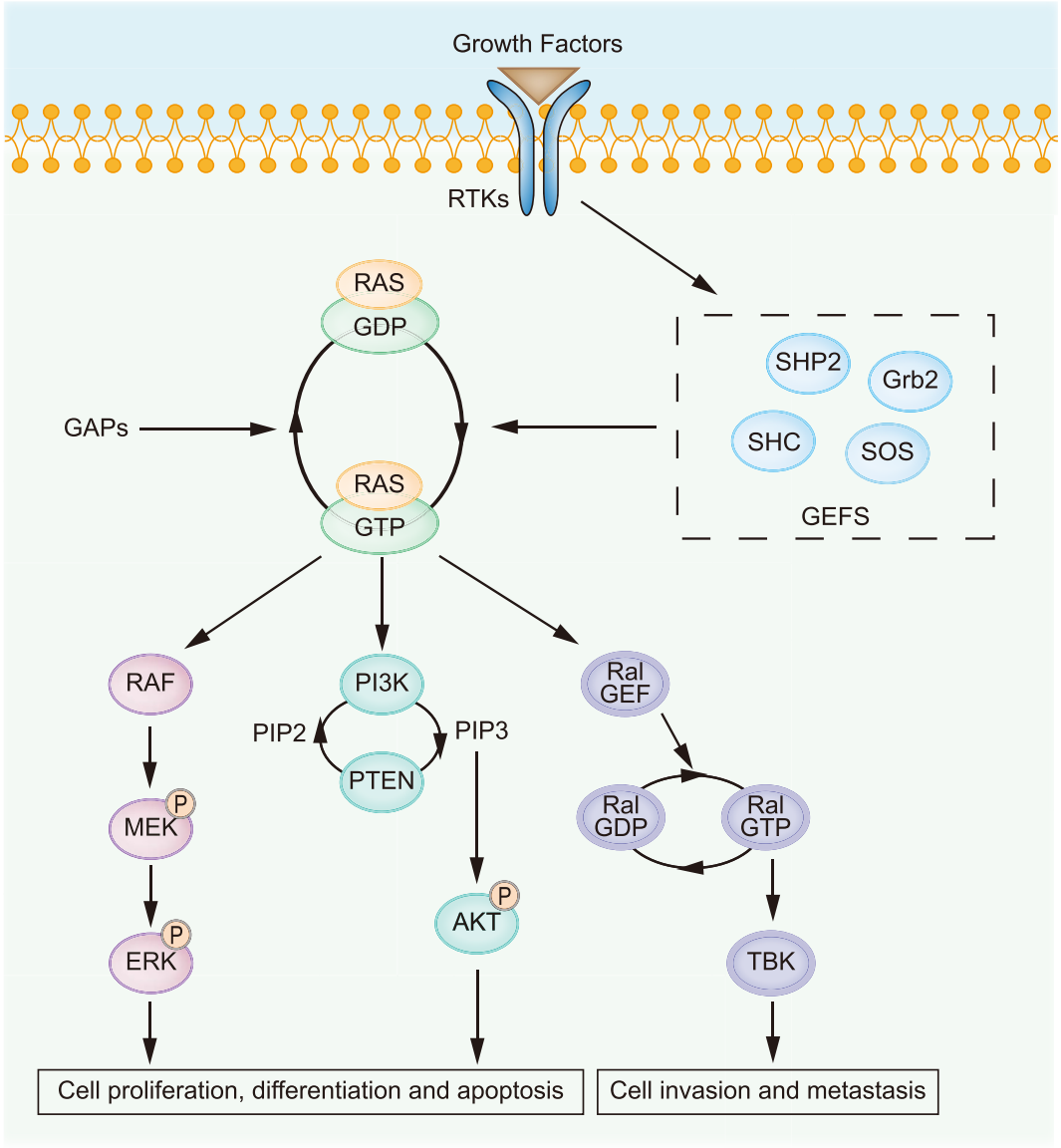

Figure 1 The Ras signaling pathway. Activated Ras by upstream RTKs or by gain-of-function mutation possesses high affinity with GTP and further activates multiple downstream signaling pathways, including Raf/MEK, PI3K/Akt and Ral/GEF cascades, which regulate important processes such as cell growth, differentiation, apoptosis, invasion, and metastasis.

multistep processes of carcinogenesis were further revealed in multiple transgenic and gene-targeted mouse models.[2] There is also strong evidence that continued expression of mutated *RAS* is necessary for tumor maintenance.[23] Suppression of Ras by RNA interference (RNAi) impaired the growth of *RAS*-mutated human cancer cells.[24] Moreover, loss of tumor protein p53 (p53), liver kinase B1 (LKB1, also called Serine—Threonine Kinase 11, STK11) or adenomatous polyposis coli (APC), which occur frequently in human cancer, enhanced tumor initiation and progression triggered by *RAS*.[25–27] Recent studies also revealed that complete knockout of either *KRAS* isoform would prevent the development of the tumor.[28] Mutated *KRAS* also regulates tumor microenvironment and in turn promotes tumor development.[29] For example, KRas reduced the production of T-cell-attracting chemokine CXCL10 and CXCL11, which was accompanied by inactivation of interferon-γ (IFN-γ) pathway and reduced antigen presentation in the tumor tissues.[30]

Despite its extreme importance in the development of tumors, direct inhibition of the mutated small GTPase has been challenging in the last few decades due to its high affinity with GTP and lack of a pocket for small molecules to bind with.[31] Therefore, mutated KRas has long been recognized as 'undruggable'. Meanwhile, strategies to target the KRas indirectly have been proposed and pursued, including inhibiting farnesyltransferase,[32] blocking downstream signaling[33–35] and exploiting the venerability of

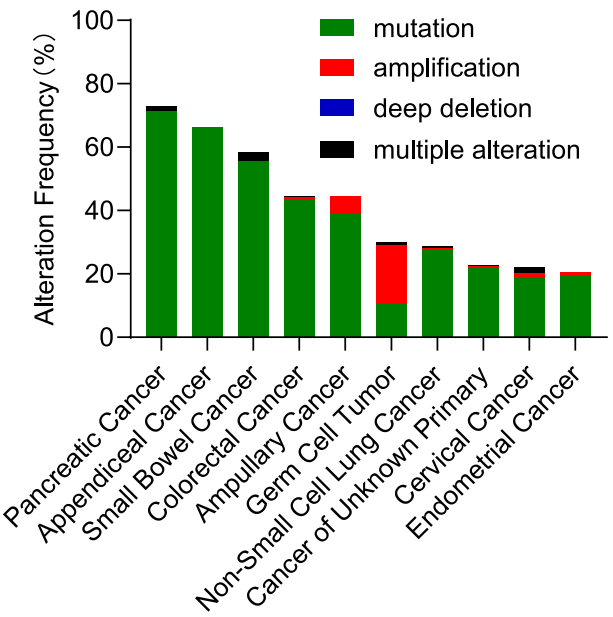

Figure 2 Frequency of *KRAS* alterations in human cancers. The 10 cancer types with most frequent *KRAS* alterations are presented. Data are derived from 13,602 samples in "Pan-Cancer Studies" analyzed by cBioPortal (www.cbioportal.org/, accessed in July 2020).

KRAS-mutated cancer via synthetic lethality.[36] Direct targeting KRas has been proposed by preventing the formation of Ras-GTP complex with GAP analogues.[37,38] The important binding sites of the KRas, including GEF binding site[39] and the binding domains with the downstream effectors,[40] are also potentially to be targeted by small molecule compounds. However, none of the strategies have been proved successful in clinical settings. ARS853 was first reported in 2016,[41] which emerged as a game changer by covalently binding to the mutated cysteine residue and selectively inhibiting the $KRas^{G12C}$. Several covalent inhibitors of $KRas^{G12C}$, such as AMG510 and MRTX849, have been rapidly developed and advanced in clinical trials. These inhibitors irreversibly bind to the switch II pocket and lock the protein in the inactive KRas-GDP state.[41–44] They offer a promising opportunity to modulate one of the major oncogenic *RAS* mutants with improved efficacy and selectivity[45] as well as reduced toxicity.[46] Remarkably, AMG510 has shown promising efficacy against patients carrying the mutation[47,48] and has been approved by U.S. Food and Drug Administration (FDA) for the treatment of locally advanced or metastatic non-small cell lung cancer (NSCLC) carrying $KRAS^{G12C}$. Moreover, combination of covalent $KRas^{G12C}$ inhibitors with other targeted therapies, immunotherapies or chemotherapies[49] are in rigorous investigation. Despite the encouraging progress, the responsive rate was not satisfying and acquired resistance is likely to develop rapidly. Nearly half of the NSCLC patients carrying $KRAS^{G12C}$ had poor response to AMG510,[47] suggesting other factors independent of KRas may affect the efficacy. Disease progression was found in patients with NSCLC, colorectal cancer and appendiceal cancer, who initially had stable disease for at least 12 weeks or an objective response to MRTX849.[50] Therefore, it is necessary to identify predictive biomarkers to stratify patients who are more likely benefitted from the therapy. Moreover, combinatorial therapy based on the elucidated mechanisms of intrinsic or acquired resistance may improve the efficacy and expand responsive population.

Advances in the covalent inhibitors of $KRas^{G12C}$

Though KRas has long been recognized as a promising target for cancer therapy, direct targeting KRas has not been feasible because of the high affinity between KRas and GTP. Alternative strategies include inhibition of the expression or post-translational modification,[3] targeting downstream effectors[29] and synthetic lethality.[51] However, none of the strategies have been successful up to date and the KRas had been considered "undruggable". Recently, small molecules that irreversibly bind to and inactivate a common oncogenic mutated $KRAS^{G12C}$ shed new light on targeting KRas.[52] These compounds selectively bind to the mutated cysteine residue of $KRas^{G12C}$ via a covalent bond and disrupt both switch-I and switch-II pocket, subverting the native nucleotide preference to GTP over GDP and impairing binding of the KRas to RAF proto-oncogene serine/threonine-protein kinase (Raf, Fig. 3).[52] Although the initial lead compounds were limited by their potency and pharmaceutical properties, the discovery and development of the covalent inhibitors have been greatly progressed and a handful of candidates including AMG510, ARS3248 and MRTX849 are currently in clinical trials (Fig. 4 and Table 1). Promising efficacy has been observed in patients with lung cancer or colorectal cancer harboring $KRAS^{G12C}$.[53] FDA has approved AMG510 (sotorasib, LUMAKRAS™) as the first and only targeted treatment for patients with $KRAS^{G12C}$-mutated locally advanced or metastatic NSCLC in May 2021.

ARS853

ARS853 was first reported in 2016, which was obtained from the modification of compound 12 and ARS107.[41] ARS853

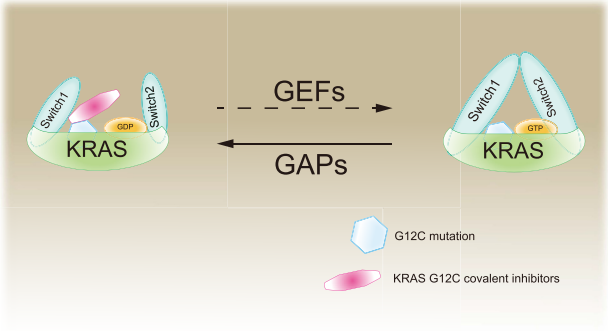

Figure 3 The mechanism of the activation of KRas and $KRas^{G12C}$ covalent inhibitors. KRas switches between active and inactive state by binding to GTP and GDP, respectively. GEFs facilitate the release of GDP, while GAPs strengthen the relatively poor intrinsic GTP enzyme activity of KRas. $KRas^{G12C}$ covalent inhibitors are mainly considered to lock $KRas^{G12C}$ in the inactive KRas-GDP state.

covalently binds to the Cys12 of mutated KRas, and extends into the Switch II pocket region located between the central β-sheet of KRas and the α2/α3 helices.[41] ARS853 may also impede the activity of KRas by interfering with GTP binding and stabilizing the bound GDP.[41] Treatment of *KRAS*^{G12C}-mutated lung cancer cells with ARS853 at 10 μM decreased KRas-GTP by over 95%, which also caused significant decrease in phosphorylation of AKT, c-Raf and ERK.[54] ARS853 inhibited the proliferation of lung cancer H358 cells harboring *KRAS*^{G12C} with a half maximal inhibitory concentration (IC_{50}) of 2.5 μM.[54] Though ARS853 is not active *in vivo*[4] which limits its further development, ARS853 is widely used as a tool compound and lays the foundation for the further development of KRas^{G12C} inhibitors.[55]

ARS1620

ARS1620 is a second-generation KRas^{G12C} inhibitor with similar characteristics to ARS853.[44] ARS1620 is a quinazoline-based compound, which is a versatile lead scaffold that is able to overcome the structure activity relationship (SAR) restrictions of ARS853 and possesses better drug-like properties.[56] ARS1620 covalently binds to Cys12 and inhibits the KRas^{G12C} activity with high potency and atropisomeric selectivity.[56] It reduced the level of KRas-GTP as well as the phosphorylation of ERK, AKT and S6 in a concentration-dependent manner and exhibited over 10 times improvement in potency compared to ARS853 to inhibit the proliferation of *KRAS*^{G12C}-mutated cancer cells.[56] ARS1620 displayed greatly improved oral bioavailability with an F value over 60% in mice compared to its parent compound of lower than 2%.[56] Matthew et al. found that ARS1620 enabled over 75% target occupancy for an extended period of time, which is necessary and sufficient to achieve therapeutic efficacy *in vivo*.[56] In fact, administration of ARS1620 at 200 mg/kg achieved tumor growth inhibition (TGI) by over 70% in mice bearing xenografts derived from 5 types of NSCLC cells respectively.[56] ARS1620 is currently in preclinical study.

AMG510

AMG510 (Sotorasib), is developed by Amgen,[15,57] which is the first KRas^{G12C} inhibitor approved by the FDA for treatment of *KRAS*^{G12C}-mutated locally advanced or metastatic NSCLC. AMG510 possesses a novel quinazolinone scaffold occupying the KRas switch II pocket and the acrylamide moiety forming a covalent bond with Cys12.[15] The aromatic ring of AMG510 is able to bind to His95, thus strengthening the interaction with the KRas^{G12C}.[42] AMG510 is highly selective for covalent modification of the KRas^{G12C} among 6451 cysteine-containing peptides profiled. Although the structure of AMG510 is similar to ARS1620, it is about 10-time more potent than the latter in inhibiting the KRas^{G12C}.[42] AMG510 inhibited KRas signaling represented by reduced p-ERK in all *KRAS*^{G12C}-mutated cell lines tested but not in cell lines harboring other types of mutations in *KRAS*.[58] AMG510 selectively inhibited the signaling and proliferation with IC_{50} values ranging from 0.010 μM to 0.123 μM in a panel of 22 cell lines tested harboring heterozygous or homozygous *KRAS*^{G12C} other than non-*KRAS*^{G12C} mutations or wild-type *KRAS*.[42] In mice bearing

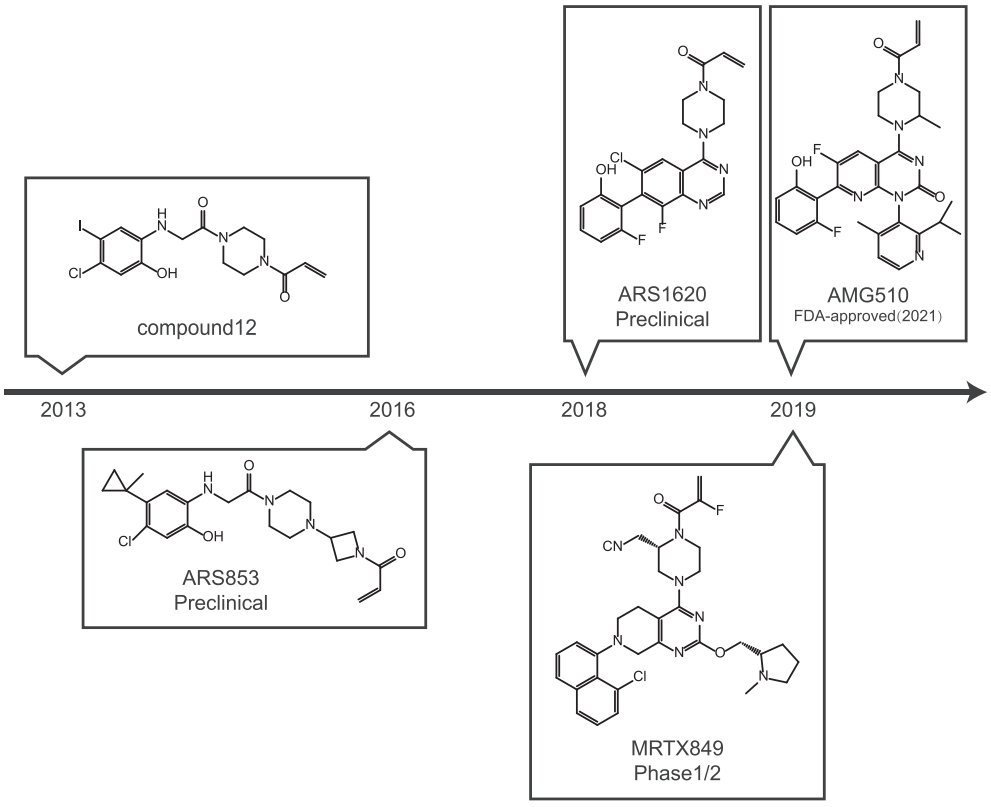

Figure 4 A chronicle of discovery and development of KRas^{G12C} covalent inhibitors.

Table 1 Registered clinical trials of $KRas^{G12C}$ inhibitors (www.clinicaltrials.gov.).

NCT Number	Phases	Status	Interventions	Conditions
NCT03600883	I/II	Recruiting	AMG 510	KRAS p.G12C Mutant Advanced Solid Tumors
NCT04006301	I	Completed	JNJ-74699157	Neoplasms|Advanced Solid Tumors|Non-small Cell Lung Cancer|Colorectal Cancer
NCT04303780	III	Recruiting	AMG 510, Docetaxel	KRAS p.G12C Mutated/Advanced Metastatic NSCLC
NCT04380753	I	Recruiting	AMG 510	Advanced/Metastatic Solid Tumors With KRAS p.G12C Mutation
NCT04625647	II	Not yet recruiting	Sotorasib	Lung Adenocarcinoma|Lung Non-Small Cell Carcinoma|Recurrent Lung Non-Squamous Non-Small Cell Carcinoma|Stage IV Lung Cancer AJCC v8|Stage IVA Lung Cancer AJCC v8|Stage IVB Lung Cancer AJCC v8
NCT04667234		Available	AMG 510	Non Small-cell Lung Cancer|Locally Advanced Unresectable NSCLC|Locally Advanced Metastatic NSCLC
NCT04685135	III	Recruiting	MRTX849, Docetaxel	Metastatic Non Small Cell Lung Cancer|Advanced Non Small Cell Lung Cancer
NCT03785249	I/II	Recruiting	MRTX849, Pembrolizumab, Cetuximab, Afatinib	Advanced Cancer|Metastatic Cancer|Malignant Neoplastic Disease
NCT04165031	I/II	Terminated	LY3499446, Abemaciclib, Cetuximab, Erlotinib, Docetaxel	Advanced Solid Tumor|Non-Small Cell Lung Cancer|Colorectal Cancer
NCT04185883	I	Recruiting	Sotorasib, PD1 inhibitor, MEK inhibitor, SHP2 allosteric inhibitor, Pan-ErbB tyrosine kinase inhibitor, PD-L1 inhibitor, EGFR inhibitor, Chemotherapeutic regimen, PD-1 inhibitor, mTOR inhibitor, CDK inhibitor, VEGF inhibitor	Advanced Solid Tumors|Kirsten Rat Sarcoma (KRAS) p.G12C Mutation
NCT04330664	I/II	Recruiting	MRTX849, TNO155	Advanced Cancer|Metastatic Cancer|Malignant Neoplastic Disease
NCT04449874	I	Recruiting	GDC-6036, Atezolizumab, Cetuximab, Bevacizumab, Erlotinib	Non-Small Cell Lung Cancer|Colorectal Cancer|Advanced Solid Tumors
NCT04585035	I/II	Recruiting	D-1553, Other	Solid Tumor, Adult|NSCLC|CRC
NCT04613596	II	Recruiting	MRTX849, Pembrolizumab	Advanced Non-Small Cell Lung Cancer|Metastatic Cancer
NCT04699188	I/II	Recruiting	JDQ443, TNO155, spartalizumab	KRAS G12C Mutant Solid Tumors|Carcinoma, Non-Small-Cell Lung|Carcinoma, Colorectal|Cancer of Lung|Cancer of the Lung|Lung Cancer|Neoplasms, Lung|Neoplasms, Pulmonary|Pulmonary Cancer|Pulmonary Neoplasms
NCT04793958	III	Recruiting	MRTX849, Cetuximab	Advanced Colorectal Cancer|Metastatic Colorectal Cancer

xenografts derived from human tumor cells, AMG510 significantly inhibited the tumor growth. AMG510 achieved regression of MIAPACA2 xenografts at the dose of 30 mg/kg, which was at least 3.3-fold lower than that of ARS1620.[42] AMG510 displayed reasonable oral bioavailability observed in all species.[15] AMG510 entered clinical trials in August 2018 due to the encouraging preclinical results and phase 3 clinical trials were initiated in March 2020 for locally advanced, unresectable or metastatic NSCLC harboring $KRAS^{G12C}$ mutation (NCT04303780). Considering that the

cancers in these patients had been refractory to previous treatments, it is valuable that a confirmed response was observed in 32.2% of the patients with NSCLC, and disease control for a few months or more was obtained in 88.1% of the patients, leading to a median progression-free survival (PFS) of 6.3 months.[59] The common side effects of AMG510 are diarrhea, nausea, vomiting, fatigue, and elevations of aminotransferase levels.[59] Up to now, no dose-limiting toxic effects have been observed even with extended treatment of AMG510. The FDA approved AMG510 (LUMAKRAS™) for the treatment of adult patients with *KRAS*^{G12C}-mutated locally advanced or metastatic NSCLC, as determined by the clinical trial in patients who have received at least one prior systemic therapy.[60] Clinical trials evaluating AMG510 as monotherapy or in combination with various agents in patients with NSCLC or other solid tumors are under way (NCT04303780 and NCT04185883).[59]

MRTX849

MRTX849 (Adagrasib) is a member of the latest generation of KRas^{G12C} inhibitors, which was developed by Mirati.[43] It irreversibly binds to mutated Cys12 in the KRas^{G12C} switch II pocket and locks it in the inactive KRas-GDP state.[43] MRTX849 showed over 1000-fold selectivity against *KRAS*^{G12C} among tested kinases with an IC_{50} of 4.7 nM to inhibit the proliferation of *KRAS*^{G12C}-mutated MIAPACA2 cells.[43] MRTX849 treatment resulted in reduction in tumor volume by more than 30% in 17 out of 26 tumor xenografts originated from lung cancer, colon cancer, pancreatic cancer, cervical cancer and esophagus cancer cells harboring *KRAS*^{G12C} mutation.[43] MRTX849 has a predicted oral bioavailability in humans of around 50% and a half-life of about 20 h in rodent and non-rodent repeat-administration toxicology studies.[61] The clinical results from the Phase 1/2 trial was first presented in 2019 AACR—NCI—EORTC meeting.[48] Among the 12 patients (6 cases of NSCLC, 4 cases of CRC, and 2 cases of appendiceal cancer) treated with MRTX849, response has been observed in 3 NSCLC patients and 1 CRC patient. The most common adverse events associated with MRTX849 were gastrointestinal side effects such as diarrhea and nausea.[6] The latest preliminary results, including updated clinical data of the drug, were announced by Mirati at the 2020 AACR—NCI—EORTC meeting.[62,63] In patients with advanced NSCLC, the overall response rate (ORR) was up to 45% (23/51) and the disease control rate (DCR) was up to 96% (49/51) across Phase 1/1b and Phase 2 clinical trials. In patients with CRC, the ORR was 17% (3/18) and the DCR was 94% (17/18) across Phase 1/1b and Phase 2 clinical trials. Four patients with pancreatic, ovarian, endometrial or cholangiocarcinoma tumors had a confirmed partial response to the therapy.[64] MRTX849 has been well tolerated as a monotherapy and in combination with BI1701963 (NCT04111458), pembrolizumab (a PD-1 inhibitor), cetuximab (an anti-EGFR antibody) and TNO-155 (a SHP-2 inhibitor).[62,63]

Challenges in the development of covalent inhibitors of KRas^{G12C}

Direct targeting the KRas^{G12C} by small-molecule covalent inhibitors represented by FDA approved AMG510 provided encouraging evidence for successful targeting the 'undruggable' KRas.[65] However, tumors harboring *KRAS*^{G12C} mutation display differential sensitivity to the inhibitors, which was reflected by ORR of 48%—50% in NSCLS patients and 6%—14% in other cancer patients.[66] Therefore, it is important to identify patients that are more likely benefitted from the treatment. As KRas sits in a complex network to regulate multiple processes of cancer, adaptive resistance has been observed in pre-clinical settings and in patients who initially responded well to the KRas^{G12C} inhibitor MRTX849.[50] Efforts have been devoted to elucidate the mechanisms leading to resistance and develop strategies to overcome the resistance.

Identification of predictive biomarkers for the efficacy of the KRas^{G12C} inhibitors

Patients with cancers harboring *KRAS*^{G12C} mutation are the most likely to respond to KRas^{G12C} covalent inhibitors. AMG510 has been shown to be active in tumor cells harboring *KRAS*^{G12C} mutation, while non-*KRAS*^{G12C} mutated cell lines were insensitive to AMG510.[42] However, the potency of AMG510 was variable among the *KRAS*^{G12C} mutated cell lines.[42] Similarly, the response is highly variable especially in colorectal cancer patients carrying *KRAS*^{G12C} mutation, which reflected the heterogeneity of cancers originated from different tissues types and even from the same tissue type.[67,68]

Recent study has reported that higher KRas-GTP level was found in the KRas^{G12C} inhibitors-sensitive cells compared to resistant cells, which was accompanied with higher score of KRas-dependency signature.[69] Considering that trapping KRas^{G12C} in a GDP-bound conformation by ARS853 is lowering its affinity for nucleotide exchange factors, there is possibility that nucleotide exchange activity modulates the effect of ARS853.[54] Indeed, *KRAS* mutations including Y40A, N116H and A146V that increase nucleotide exchange attenuated the effect of ARS853 on KRas^{G12C}-GTP, while *KRAS*^{Y32S} caused a slight augmentation in its effect. Therefore, co-occurrence of mutations in KRas that potentiates nucleotide exchange would reduce the potency of KRas^{G12C} inhibitors.[54] A recent phase II clinical trial found that improved efficacy with AMG510 was seen in patients concurrently carrying mutated STK11 and wild-type KEAP1 ($n = 22$) with median PFS of 11.0 months and median overall survival (OS) of 15.3 months.[70] However, Hallin J et al. demonstrated the heterogeneity and complexity of *KRAS*-mutated cancers and suggested that individual binary biomarkers might not be able to predict therapeutic response.[43] Further studies are warranted in both preclinical and clinical settings.

Identification of biomarkers to monitor the efficacy of KRas^{G12C} inhibitors

Important downstream effectors of active KRas, including p-ERK, p-AKT and p-S6, have been proposed to be potential biomarkers to monitor the efficacy of KRas^{G12C} inhibitors. The decrease of p-ERK could be observed in most *KRAS*^{G12C} cell lines after being treated with KRas^{G12C} inhibitors. A recent study revealed the decrease in p-ERK correlated well with occupancy of the KRas^{G12C} by AMG510 in

H358 cells.[42] The level of target genes of ERK, such as dual-specificity phosphatases (*DUSP*) decreased after KRas^{G12C} inhibitor treatment,[43,44,68] which could also be considered as a potential biomarker candidate. AKT is an intermediate effector downstream of KRas via PI3K signaling pathway. However, suppression of phosphorylated AKT was only observed in a few lines of NSCLC cells harboring *KRAS^{G12C}* mutation.[42,44] It seems that most of the *KRAS^{G12C}* mutated cells have distinctive mechanisms to activate PI3K pathway and p-AKT may not be able to serve as a biomarker for the efficacy.[44,68] Meanwhile, S6 is the integrated signaling node of both MAPK and PI3K pathways, indicating a potential indicator of KRas inhibition. Sustained shutdown of p-S6 may be a good predictor of the effective suppression of cell viability.[71] Patricelli et al. observed reduced level of p-S6 after treatment with ARS853 for both short time and long time[41] and similar phenomenon was observed after MRTX849 treatment,[43] which indicated that the level of p-S6 might potentially monitor the efficacy. However, the outcomes were controversial in the experiments with ARS1620.[44] Similar to p-AKT, the level of p-S6 was not affected in most cells after treated with ARS1620.[44] It appears that alternative biomarkers instead of afore mentioned in the signaling pathway need to be discovered to better monitor the efficacy of KRas^{G12C} inhibitors.

Strategies to overcome resistance to KRas^{G12C} inhibitors

Though the clinical response is encouraging for the KRas^{G12C} inhibitors, the efficacy could be circumvented by both intrinsic and adaptive resistance. Indeed, disease progression was found in patients with NSCLC, CRC and appendiceal cancer, who initially had stable disease for at least 12 weeks or an objective response to MRTX849.[50] Alternative KRas mutation or high-level amplification of the KRas^{G12C} allele, activation of upstream RTKs, downstream effectors and other isoforms in Ras family could lead to resistance to KRas^{G12C} inhibitors, and co-targeting these pathways and KRas^{G12C} might be potential strategies to overcome the resistance and improve the efficacy of KRas^{G12C} inhibitors.

Co-targeting upstream signaling

As KRas sits in the center of RTK-mediated signaling, bypass the Ras activity via activation of upstream may result in resistance to KRas^{G12C} inhibitors. The clinical studies showed that the ORR of MRTX849 or AMG510 was 48%—50% in patients with *KRAS^{G12C}* mutated NSCLC, and only 6%—14% in patients with other types of cancer,[66] indicating that these patients are usually intrinsically resistant to KRas^{G12C} inhibitors. To identify mechanism underlying intrinsic resistance to KRas^{G12C} inhibitors in CRC patients, Amodio et al. measured the levels of active RTKs in colorectal cancer and NSCLC cell lines using p-RTK arrays and found enhanced activation of EGFR in CRC cell lines.[67] Consistently, combination of an EGFR inhibitor with a KRas^{G12C} inhibitor achieved tumor regression in CRC patient-derived xenograft (PDX).[67]

In addition to intrinsic hyper-activation of EGFR in CRC cells, adaptive activation of multiple RTKs, including EGFR, human epidermal growth factor receptor-2 (HER2), and fibroblast growth factor receptor (FGFR) was detected in multiple *KRAS^{G12C}*-mutated cells treated with ARS1620 for 48 h in a highly heterogeneous pattern.[68] For instance, primary dependency on EGFR family signaling was found in SW1463 cells, while MIAPACA2 cells appeared to be more dependent on FGFR signaling than EGFR/HER family members. Moreover, rapid but non-uniform adaptation to ARS1620 was detected using scRNA-seq at a single-cell resolution. Similarly, MET amplification, activating mutations in RET and oncogenic fusions involving ALK, RET and FGFR3 were found in patient relapsed during the treatment of MRTX849.[50] Although targeting a dominant RTK-driven feedback reactivation is an attractive strategy to overcome adaptive resistance, it is difficult to propose a uniform drug combination as RTKs were activated heterogeneously across cancers carrying *KRAS^{G12C}* mutation. SHP2 is a primary effector of receptor tyrosine kinase signaling, which dephosphorylates Ras and results in increased Ras—Raf association and activation of downstream MAPK signaling.[72] In this scenario, the RTK-associated phosphatase SHP2 may represent a common target to inhibit feedback reactivation of multiple RTKs and improve the efficacy across heterogeneous *KRAS^{G12C}*-mutated cancers. Indeed, co-targeting KRas^{G12C} with a SHP2 inhibitor showed synergistic effect both *in vitro* and *in vivo*, which blocked downstream signals and finally led to tumor regression.[68,69,73] These studies indicated that RTK activation could lead to the resistance to KRas^{G12C} inhibitors, and co-targeting RTK or SHP2 and KRas^{G12C} could circumvent the resistance. In fact, the combination of SHP2 inhibitors or EGFR inhibitors with KRas^{G12C} inhibitors are currently under clinical investigation in *KRAS^{G12C}* mutated solid tumors including lung cancer and colorectal cancer (Table 1).

Co-targeting downstream signaling

MAPK pathway, which sits downstream of KRas, is a key regulator of cell proliferation, survival and differentiation.[74,75] The effectors of MAPK pathway, MEK1/2 and ERK1/2, were initially robustly inactivated in all cell lines after ARS1620 treatment, while phosphorylation of MEK and ERK restored upon prolonged treatment.[44] Moreover, activating mutation in *MAP2K1* and oncogenic fusions in *RAF1* were found in clinical samples with adaptive resistance to MRTX849.[50] It has been shown that combination of a KRAS^{G12C} inhibitor with a MEK inhibitor could drive tumor regression,[46] which indicated this strategy may overcome the resistance led by the rebound of MEK.

Sustained activation of another important parallel signaling, PI3K-AKT pathway, may also mediate the resistance to KRas^{G12C} inhibitors. PI3K pathway is often aberrantly activated in *KRAS^{G12C}* cell lines. Inhibition of *KRAS^{G12C}* led to decreased p-AKT in only 3 of the 12 cell lines, suggesting that PI3K pathway may not be regulated by KRas alone in most *KRAS* mutated cells.[44] Moreover, loss-of-function mutations in PTEN were found in clinical samples with acquired resistance to MRTX849,[50] which may also lead to activation of PI3K signaling. Accordingly, concurrent inhibition of KRas^{G12C} and PI3K improved the efficacy and achieved tumor regression.[44] mTOR is a crucial downstream of both PI3K and MAPK pathways. However, transiently reduced p-S6 was observed in few cell lines after treated

with KRas^{G12C} inhibitors.[44] The sustained mTOR activation indicates that mTOR activity might be independent of KRas. Sandra et al. performed a high-throughput combinatorial drug screening to evaluate the synergy of ARS1620 in combination with a panel of 112 small molecules of high clinical relevance and founded that inhibitors targeting PI3K and mTORC1 pathway were the most synergistic with ARS1620.[44] Similarly, the combination of an mTOR inhibitor with a KRas^{G12C} inhibitor could significantly reduce the level of both p-S6 and p-ERK, which may overcome the resistance to KRas^{G12C} inhibitors.[46] AMG510 in combination with MEK inhibitor or mTOR inhibitor is being tested in clinical trials (NCT04185883, Table 1).

The Ras-MAPK pathway plays an important role in cell cycle transition. Previous studies have identified CDK1 and CDK4 as synthetic lethal targets in *KRAS*-mutated CRC and NSCLC respectively,[76,77] indicating that cell cycle regulators might be potential targets for tumors harboring *KRAS* mutation. Cyclin D1-CDK4/6 complex phosphorylates the tumor suppressor RB, resulting in the release of E2F and transcription of genes involved in G1-S transition. To identify potential combination therapies to overcome the primary resistance to KRas inhibitors, high-throughput screenings have been performed and found that signaling adaptation could limit the efficacy of KRas^{G12C} inhibitors and combination with CDK4/6 inhibitors overcame this resistance.[78] A recent study revealed that the expression of aurora kinase A (AURKA), a serine/threonine kinase regulating mitosis by binding to the centrosome, was initially downregulated when *KRas^{G12C}* mutated gastrointestinal (GI) cancer cells were treated with KRas^{G12C} inhibitors but rebounded after long-term treatment.[79] Simultaneous inhibition of AURKA and KRas^{G12C} displayed synergistic anti-proliferative activity. Thus targeting AURKA may potentially be a novel therapeutic strategy for treating *KRAS*-mutated GI cancer.[80] CDK inhibitors in combination of AMG510 are currently tested in clinical trials (Table 1).

Co-targeting alternative pathways

There are three members in the *RAS* family and they perform distinctive activity in cells. Rapid adaptive feedback reactivation in Ras pathway has been observed in the majority of *KRAS^{G12C}* mutated cancer cells upon treatment of ARS1620 or AMG510.[68] The activity of NRas and HRas was revealed to increase in *KRAS^{G12C}* mutated cells treated with ARS1620, which could not be inhibited by KRas^{G12C}-specific inhibitors and might attenuate the activity of KRas^{G12C} inhibitors.[68] Moreover, acquired *KRAS* alterations including G12D/R/V/W, G13D, Q61H, R68S, H95D/Q/R, Y96C, and high-level amplification of the KRas^{G12C} allele were found in patients developed acquired resistance to MRTX849.[50] Introduction of the clinically observed switch II pocket mutations (R68S, H95D, H95Q, H95R, and Y96C) conferred marked resistance to MRTX849,[50] which may contribute to clinical resistance to KRas^{G12C} inhibitor. A novel clinical *KRAS^{Y96D}* mutation has also been reported to affect the binding of MRTX849 to the switch-II pocket, and thus confers resistance to KRas^{G12C} inhibitors.[81] Though pan-Ras inhibitors might overcome the resistance,[82] the related toxicity should be concerned. The next-generation KRas covalent inhibitors are warranted to be discovered.

The epithelial-mesenchymal transition (EMT) is a process that epithelial cells lose cell polarity as well as cell-cell adhesion to become mesenchymal cells with gained migratory and invasive properties.[83] The EMT is considered a key driver of invasion and metastasis in tumor for a long time, while recent studies revealed its role in mediating resistance to chemotherapy.[84] Similarly, EMT was observed in cells with acquired resistance to AMG510. Moreover, induction of EMT by TGF-β rendered sensitive cells resistant to KRas^{G12C} inhibitors.[69] Therefore, EMT might mediate resistance to KRas^{G12C} inhibitors and targeting EMT might enhance the efficacy.

KRas may have profound influence on tumor immune microenvironment such as allowing tumor cells to escape the anti-tumor immune response and thus promoting the growth and metastasis of tumor cells.[85,86] Recent study has reported that the combination of AMG510 and immunotherapies achieved improved efficacy compared to monotherapy. For example, combination of AMG510 and anti-PD-1 antibody led to complete regression in nine out of ten mice tested and generated T cell memory.[42] AMG510 induced an inflammatory microenvironment by increasing tumor infiltration of macrophages and dendritic cells as well as the expression of MHC class I antigens on tumor cells.[42] The combination of KRas^{G12C} inhibitors and PD-1/PDL1 antibodies is being tested in clinical trials (Table 1).

Perspectives

KRAS is one of the most frequently mutated oncogenes in human cancer and is identified as an attractive target for cancer therapy. However, KRas protein was considered undruggable until the approval of AMG510 for treating patients with *KRAS^{G12C}*-mutated locally advanced or metastatic NSCLC. These covalent KRas^{G12C} inhibitors trap the protein in the inactive GDP-bound state, which represents a major step towards targeting the undruggable KRas and provided valuable insights into targeting other common *KRAS* mutations such as G12D, G13C, or G13D.[87] Encouragingly, Mirati reported the promising preclinical activity of a potential first-in-class G12D selective inhibitor, MRTX1133, with similar features of covalent *KRAS^{G12C}* inhibitors.[62,63]

Although early-phase clinical trial results of AMG510 and MRTX849 among NSCLC patients are promising, the objective response rate is around 50%, which indicates around half NSCLC patients harboring *KRAS^{G12C}* mutation don't respond to the treatment, and the activity of AMG510 and MRTX849 in patients with colorectal cancer and appendiceal cancer appears to be less favorable.[66] In addition to *KRAS^{G12C}* mutation, additional biomarkers were needed to stratify patients. Reliable biomarkers also need to be investigated in the future clinical trials to monitor the efficacy. Moreover, the mechanisms of response or resistance should be comprehensively elucidated in both preclinical and clinical settings. It has been reported that activation of upstream RTKs and SHP2, downstream MEK, AKT and mTOR, as well as alternative mutations in KRas would mediate resistance to KRas^{G12C} inhibitors. Indeed, combination of KRas^{G12C} inhibitors with RTK inhibitors, SHP2 inhibitors, MEK inhibitors or mTOR inhibitors is currently tested in clinical trials (Table 1). In particular, activated Ras may possess profound influences on tumor

immune microenvironment such as allowing cancer cells to evade the antitumor immune response.[85] Recent studies found that the combination of a $KRas^{G12C}$ inhibitor and an anti-PD-L1 antibody displayed enhanced efficacy *in vivo*.[42] Better understanding the mechanisms of action of $KRas^{G12C}$ inhibitors on tumor cells and the tumor micro-environment would be helpful to optimize the therapy based on $KRas^{G12C}$ inhibitors.

In summary, the emerging $KRas^{G12C}$ inhibitors have provided great opportunities for the treatment of *KRAS*-mutated cancers. It is still of great importance to improve the clinical efficacy through identifying predicting biomarkers and proposing rational drug combinations to circumvent drug resistance.

Author contributions

Ling-hua Meng and Yu-xiang Wang conceived the project and revised the manuscript. Hui-yu Li and Wei-liang Qi summarized the literature and composed the manuscript. All authors proofread the manuscript and approved to submit the final manuscript.

Conflict of interests

The authors declare no competing interests.

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