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Drugging K-Ras^{G12C} through covalent inhibitors: Mission possible?

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ABSTRACT

Ras, whose mutants are present in approximately 30% of human tumours, is one of the most important oncogenes. Drugging Ras is thus regarded as the quest for the Holy Grail in cancer therapeutics development. Despite more than three decades of efforts, drug discovery targeting Ras constantly fails, rendering Ras undruggable, due to its smooth surface and picomolar affinity towards guanosine substrates. The most frequently mutated isoform of Ras is K-Ras, accounting for >85% of Ras-driven cancers, and one majority of them is the G12C mutation. Recent advances in structural biology shed light on drugging Ras, and one of the cutting-edge breakthroughs is the design of covalent G12C-specific inhibitors targeting the mutated cysteine. This type of inhibitor can be classified into substrate-competitive orthosteric inhibitors and non-competitive allosteric inhibitors. They display improved selectivity and enhanced potency due to their G12-specific and irreversible covalent binding nature. Thus, they represent a new hope for revolutionizing the conventional characterization of Ras as "undruggable" and pave a promising avenue for further drug discovery. Here, we provide comprehensive structural and medicinal chemical insights into K-Ras covalent inhibitors specific for the G12C mutant. We first present an in-depth analysis of the conformations of the inhibitor binding pockets. Then, all the latest covalent ligands selectively inhibiting K-Ras^{G12C} are reviewed. Finally, we examine the current challenges faced by this new class of anti-Ras inhibitors.

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1. Introduction

Ras is a small GTPase mediating various cellular signalling pathways and is implicated in cell growth and survival (Barbacid, 1987; Bryant, Mancias, Kimmelman, & Der, 2014; Cox & Der, 2010; Karnoub & Weinberg, 2008; Malumbres & Barbacid, 2003). The Ras genes encode H-Ras, N-Ras and K-Ras (Colicelli, 2004; Rojas, Fuentes, Rausell, & Valencia, 2012). They function as binary switches in signalling transduction such as the Ras-Raf-MEK-ERK and the Ras-PI3K-Akt pathways (Boriack-Sjodin, Margarit, Bar-Sagi, & Kuriyan, 1998; Li, Jang, Zhang, & Nussinov, 2018; Lu et al., 2016; Lu, Jang, Gu, Zhang, & Nussinov, 2016; Margarit et al., 2003; Moodie, Willumsen, Weber, & Wolfman, 1993; Najem et al., 2017; Ni, Liu, Zhang, & Lu, 2018; Nussinov et al., 2017; Rodriguez-Viciana et al., 1997; Rodriguez-Viciana & Downward, 2001; Rubio, Rodriguez-Viciana, Downward, & Wetzker, 1997; Thomsen

Abbreviations: IC50, half-maximum effective concentration; PPI, protein-protein interaction; SAR, structure-activity relationship; S-IIP, switch II pocket; SW1, switch I; SW2, switch II.

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et al., 2017; Vojtek, Hollenberg, & Cooper, 1993; Wood, Sarnecki, Roberts, & Blenis, 1992) through cycling between the GDP-bound inactive state and the GTP-bound active state (Downward, 1990; Grand & Owen, 1991; Takai, Sasaki, & Matozaki, 2001; Vetter & Wittinghofer, 2001; Wittinghofer & Pai, 1991). In 1982, mutationally activated Ras genes were first identified in human cancers (Chang, Gonda, Ellis, Scolnick, & Lowy, 1982; Jemal et al., 2009; Karnoub & Weinberg, 2008), and currently, according to the Catalogue of Somatic Mutations in Cancer (COSMIC) (Forbes et al., 2011), Ras mutations account for approximately 30% of human cancers, encompassing lung, colon, and pancreatic cancers (Eser, Schnieke, Schneider, & Saur, 2014; Holderfield, Deuker, McCormick, & McMahon, 2014; Huang et al., 2018; Jiang et al., 2017; Krens, Baas, Gelderblom, & Guchelaar, 2010; Lindsay, Jamal-Hanjani, Forster, & Blackhall, 2018; Mann, Ying, Juan, Jenkins, & Copeland, 2016; McCormick, 2015a, 2015b; Prior, Lewis, & Mattos, 2012; Shen et al., 2017; Spindler et al., 2015). Among them, K-Ras mutations are found in approximately 85% of all Ras-driven cancers and are thus regarded as the most frequently mutated oncogene in human cancer (McCormick, 2015a; Ostrem & Shokat, 2016; Peeters et al., 2015). Ras is therefore established as one of the most important targets in oncology, and its inhibitors are regarded as a promising direction in anticancer drug development (Cox, Fesik, Kimmelman, Luo, & Der, 2014; Holderfield, 2018; Lu, Jang, Gu, et al., 2016; McCormick, 2015a; Nussinov et al., 2016; Ostrem & Shokat, 2016; Papke & Der, 2017; Quah, Tan, Teh, & Stanslas, 2016; Rao et al., 2018; Sautier, Nising, & Wortmann, 2016; Sebti & Hamilton, 1997). Previous attempts to tackle oncogenic Ras include low-affinity direct orthosteric inhibitors such as SCH-53239 (Peri et al., 2005; Taveras et al., 1997), protein-protein interaction (PPI) inhibitors for Ras and guanine nucleotide exchange factors (GEFs) such as DCAI (Hocker et al., 2013; Lu, Jang, Zhang, & Nussinov, 2016; Maurer et al., 2012; Ni, Lu, & Zhang, 2019; Sun et al., 2012), and inhibitors of Ras-effector PPIs such as Kobe 0065 (Herrmann et al., 1998; Kidger, Sipthorp, & Cook, 2018; Quevedo et al., 2018; Shima et al., 2013). Additionally, targeting Ras by disrupting its cellular localization or post-translational modification is now under vigorous development (Chen, Makarewicz, Knauf, Johnson, & Fagin, 2014; Gana-Weisz et al., 1997; Sebti & Hamilton, 1997; Y. Zhou, Prakash, Gorfe, & Hancock, 2018; Zimmermann et al., 2013). However, despite sustained attempts for more than thirty years, none of these efforts have successfully tamed Ras for clinical therapeutics, and new methodology for anti-Ras drug discovery is needed.

One of advances in drugging Ras is the discovery of allele-specific inhibitors. The vast majority of Ras oncogenic mutations are concentrated on several hot spot residues (Forbes et al., 2011; Lu et al., 2015; Lu, Jang, Nussinov, & Zhang, 2016; Prior et al., 2012), especially G12. For example, K-Ras G12C accounts for >59% of all cases of non-small cell lung cancer (NSCLC), and it makes up 12% of all K-Ras mutations in cancers (Cox et al., 2014; Forbes et al., 2011; Visscher, Arkin, & Dansen, 2016). Hence, advances in the design of K-Ras^{G12C}-selective covalent inhibitors (Fell et al., 2018; Janes et al., 2018; Lim et al., 2014; Ostrem, Peters, Sos, Wells, & Shokat, 2013; Patricelli et al., 2016; Wijeratne et al., 2018; Xiong et al., 2017; Zeng et al., 2017) shed light on anti-Ras drug development and personalized precision cancer therapeutics. Mutant-selective K-Ras inhibitors exploit the reactive and nucleophilic cysteine at position No. 12. Through modification with a disulfide bond, these covalent binders can either project into the allosteric pockets and inhibit K-Ras activity allosterically or extend into the orthosteric substrate site and compete with GDP/GTP for inhibition. The trend of allosteric K-Ras^{G12C} covalent inhibitors started with the discovery of compounds **6** and **12** (hereafter referred to as the 6H05 series) and the identification of their corresponding novel allosteric site, the switch II pocket (S-IIP), by Ostrem et al. (2013). Following this stream, a plethora of more potent compounds have been developed afterwards, such as the quinazoline series (Zeng et al., 2017), the ARS series (Janes et al., 2018; Patricelli et al., 2016; Wijeratne et al., 2018) and the tetrahydropyridopyrimidines series (Fell et al., 2018). Regarding covalent orthosteric G12C K-Ras inhibitors, they are exemplified by the SML series developed by Gray's group (Lim et al., 2014; Xiong et al., 2017). Covalent modifiers feature potential pharmacological advantages and prolonged duration of drug action (Baillie, 2016; Bauer, 2015; Bradshaw et al., 2015; Christensen et al., 2014; Lu & Zhang, 2017; Nolte et al., 2014; Wang, Lodge, Fierke, & Mapp, 2014). Thus, they favour enhanced potency and lower dosage for administration. Moreover, given their mutant-specific binding modes, covalent inhibitors exhibit higher selectivity, thereby enabling the possibility of more precise cancer therapeutics (Baillie, 2016; Bauer, 2015; Bradshaw et al., 2015; Christensen et al., 2014; Lu & Zhang, 2017; Visscher et al., 2016; Wang, Lodge, et al., 2014). As such, the development of K-Ras^{G12C}-specific covalent inhibitors represents a prospective strategy in the campaign to attack the "intractable" Ras and supplies a novel paradigm for future related anticancer drug design.

Here, we review the current status of the development of G12C K-Ras mutant-specific covalent inhibitors. We first summarize all the reported covalent inhibitors and analyse the structural details of their binding pockets. Then, we categorize the latest advances in allosteric and orthosteric ligands and provide mechanistic insights into their functional modes, biochemical effects and potential applications in future drug discovery. Moreover, obstacles, possible solutions and future directions for this type of novel anti-Ras therapeutics are also discussed. Our study will provide valuable guidance for the design of K-Ras mutant-specific inhibitors and related precision medicine for cancer drug discovery in the future.

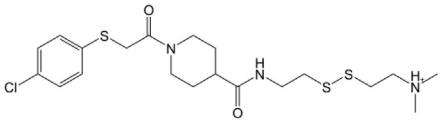
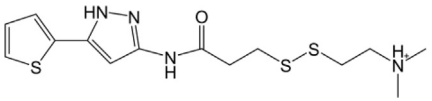
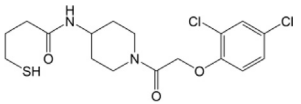
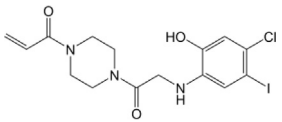
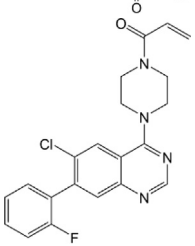
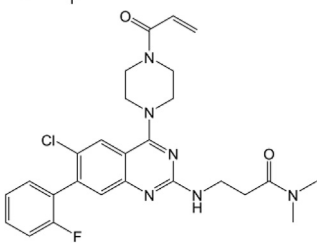
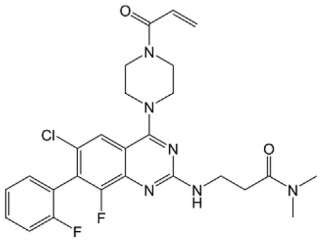
2. Solved co-crystal structures of K-Ras^{G12C} and its mutant specific covalent inhibitors

To date, five series of 20 K-Ras^{G12C} allele-selective inhibitors or related parent cores have been reported, and seven of them have been co-crystallized with the K-Ras protein (Table 1) (Fell et al., 2018; Hunter et al., 2014; Janes et al., 2018; Lim et al., 2014; Ostrem et al., 2013; Xiong et al., 2017; Zeng et al., 2017). All these ligands can be classified as allosteric or orthosteric inhibitors. For the former, their binding pockets do not overlap or interfere with the guanosine nucleotide substrate site. These allosteric ligands mainly function by either altering the K-Ras protein structure or disrupting its PPIs with nucleotide exchange factors such as SOS, thereby capturing K-Ras in a GDP-bound inactive state (Hansen et al., 2018; Janes et al., 2018; Lito, Solomon, Li, Hansen, & Rosen, 2016; Ostrem et al., 2013; Zeng et al., 2017). For the latter, they project into the active site on K-Ras and compete with GTP/GDP substrates for binding. Through orthosteric targeting, they disrupt substrate loading, trap the protein in an inactive conformation, and exert an inhibitory effect (Hunter et al., 2014; Lim et al., 2014; Xiong et al., 2017).

The six structures of G12C K-Ras in complex with covalent allosteric inhibitors include 4LUC (PDB ID) for compound **6** reported by Ostrem et al. (2013), 5V9L and 5V0O for **1_AM** and **3_AM** reported by Zeng et al. (2017), 5F2E for ARS-853 reported by Patricelli et al. (2016), 5V9U for ARS-1620 reported by Janes et al. (2018) and 6N2K for compound **12** reported by Fell et al. (2018). The only crystal structure of K-Ras^{G12C} in complex with a covalent orthosteric inhibitor is 4NMM for SML-8-73-1 reported by Hunter et al. (2014).

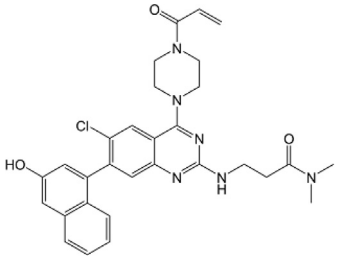
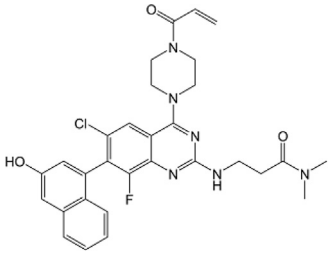
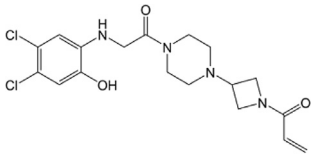
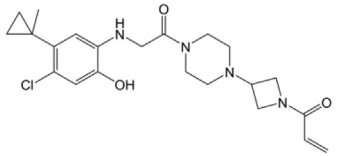
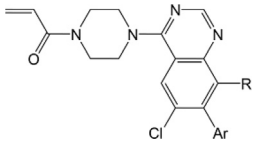
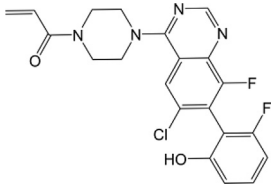
Superposition of these inhibitor-bound structures revealed the different locations and topology of the pockets for allosteric or orthosteric ligands (Fig. 1). For allosteric inhibitors, their binding sites mainly adopt linear conformations, projecting away from the mutated cysteine. They situate above the central β-sheets and lie between the switch II (SW2) and α3 helix. The different binding poses of allosteric ligands highlight the relatively flexible nature of allosteric pockets. Moreover, the quinazoline and tetrahydropyridopyrimidines series ligands (Fell et al., 2018; Zeng et al., 2017), which exhibit enhanced efficacy and selectivity, extend their additional substituents outside the cavity and make extra contact with the adjacent α3 helix. These findings suggest that the allosteric pockets and their proximal structures are relatively dynamic and

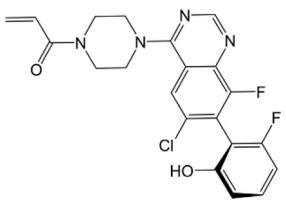
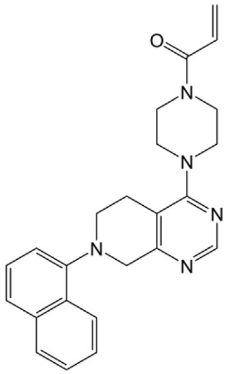
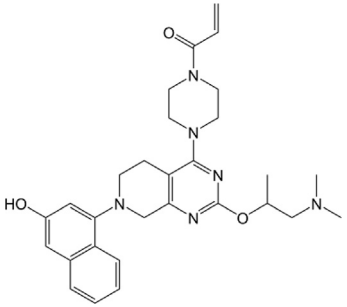
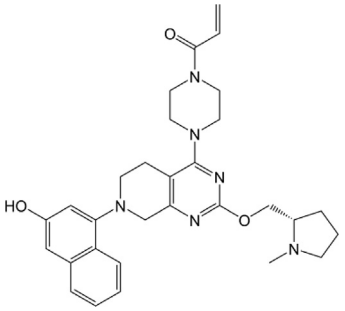
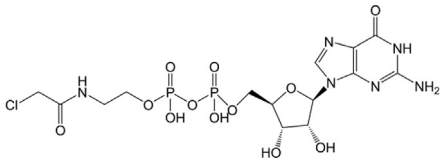
Table 1
Current reported K-Ras^{G12C}-specific covalent inhibitors and scaffolds.

Compound names[a]	Series names	Compound chemical structures	Compound ID in article	Inhibition Mechanism	Binding pocket	PDB ID	Refs
6H05	6H05 series		**1**	Allosteric	–	–	(Ostrem et al., 2013)
2E07	6H05 series		**2**	Allosteric	–	–	(Ostrem et al., 2013)
Compound **6**^{Ostrem}	6H05 series		**3**	Allosteric	7,9-14,16,34,35,56,58-63,68,71,72,78,86,96,97,99,100	4LUC	(Ostrem et al., 2013)
Compound **12**^{Ostrem}	6H05 series		**4**	Allosteric	–	–	(Ostrem et al., 2013)
Compound **1**^{Zeng}	Quinazoline series		**5**	Allosteric	–	–	(Zeng et al., 2017)
1_AM	Quinazoline series		**6**	Allosteric	9–14,16,34,58-61,68,72,78,86,92,95,96,99,100,103,	5V9L	(Zeng et al., 2017)
2_AM	Quinazoline series		**7**	Allosteric	–	–	(Zeng et al., 2017)

(continued on next page)

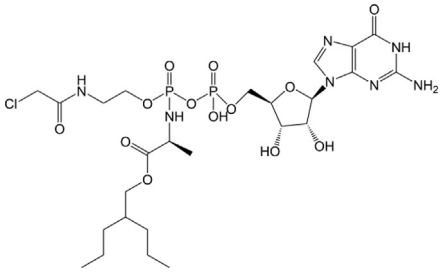
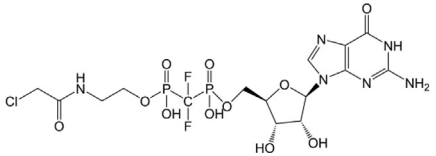
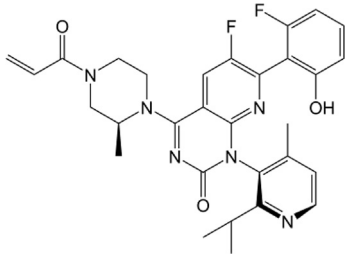
Table 1 (*continued*)

Compound names[a]	Series names	Compound chemical structures	Compound ID in article	Inhibition Mechanism	Binding pocket	PDB ID	Refs
3_AM	Quinazoline series		**8**	Allosteric	9–14,16,34,58-65,68,69,72,78,86,88,92,95,96,99,100,102,103	5V9O	(Zeng et al., 2017)
4_AM	Quinazoline series		**9**	Allosteric	–	–	(Zeng et al., 2017)
ARS-107	ARS series		**10**	Allosteric	–	–	(Patricelli et al., 2016)
ARS-853	ARS series		**11**	Allosteric	7,9-14,16,34,37,58-65,68,69,72,78,86,96,99,100,102,103,	5F2E	(Patricelli et al., 2016)
ARS-scaffold	ARS series		**12**	Allosteric	–	–	(Wijeratne et al., 2018)
Compound **1**[Wijeratne]	ARS series		**13**	Allosteric	–	–	(Wijeratne et al., 2018)

ARS-1620	ARS series		**14**	Allosteric	9–14,16,34,58-63,68,69,72,86,95,96,99,100,102,103,	5V9U	(Janes et al., 2018)
Compound **4**[Fell]	Tetrahydropyridopyrimidines series		**15**	Allosteric	–	–	(Fell et al., 2018)
Compound **12**[Fell]	Tetrahydropyridopyrimidines series		**16**	Allosteric	9–14,16,34,58-65,68,69,72,88,92,95,96,99,100,102,103	6N2K	(Fell et al., 2018)
Compound **13**[Fell]	Tetrahydropyridopyrimidines series		**17**	Allosteric	–	–	(Fell et al., 2018)
SML-8-73-1	SML series		**18**	Orthosteric	10–19,28-36,57-60,86,116-120,144–147	4NMM	(Hunter et al., 2014; Lim et al., 2014)

(continued on next page)

Table 1 (*continued*)

Compound names[a]	Series names	Compound chemical structures	Compound ID in article	Inhibition Mechanism	Binding pocket	PDB ID	Refs
SML-10-70-1	SML series		**19**	Orthosteric	–	–	(Xiong et al., 2017)
Compound **11**[Xiong]	SML series		**20**	Orthosteric	–	–	(Xiong et al., 2017)
AMG 510 (US20180177767)[b]	–		**21**	Allosteric	–	–	–

[a] The names of researchers that report the compounds were added to differentiate similar numberings among different inhibitor series.
[b] AMG 510 (**21**) is a patented compound developed by Amgen Inc.

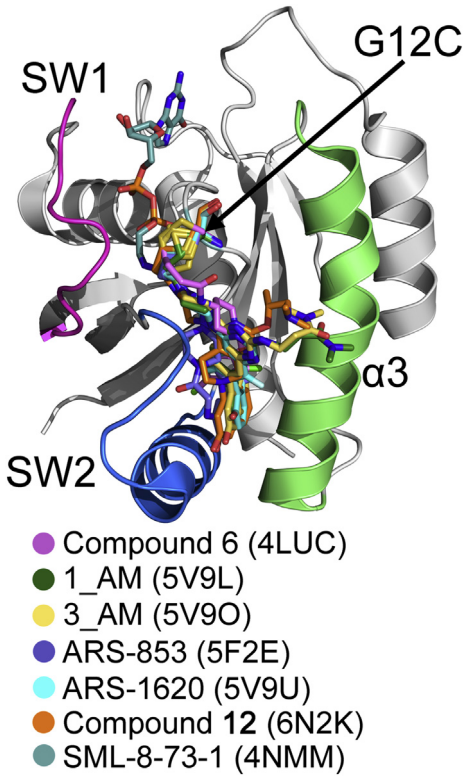


Fig. 1. Multiplicity of binding modes of all current K-Ras^{G12C}-specific covalent inhibitors. The representative crystal structure of G12C K-Ras in complex with SML-8-73-1 (PDB ID: 4NMM) is selected as the template, and all other structures of K-Ras^{G12C} allele-specific inhibitors are aligned to that template. The overall backbone structure is coloured in gray, compound **6** (4LUC) is shown in pink, **1_AM** (5V9L) is shown in green, **3_AM** (5V9O) is shown in pale yellow, ARS-853 (5F2E) is shown in blue, ARS-1620 (5V9U) is shown in cyan, compound **12** (6N2K) is shown in orange, and SML-8-73-1 (4NMM) is shown in dark blue. Important structural elements of the switch I (SW1) (magenta), the switch II (SW2) (blue), and the α3-helix (green) in K-Ras are also highlighted.

elastic and that their conformations can be induced and altered through medicinal chemical efforts. Such characteristics emphasize the great prospects of the allosteric sites for future drug design and the potential opportunity to improve the potency and specificity of the compounds by introducing more interactions with the neighbouring structures. The orthosteric pocket resides opposite the allosteric site. Orthosteric inhibitors insert into this site and display the same conformation as GDP, which implies that this cavity is relatively rigid and may limit subsequent lead optimization. Hence, compared with the orthosteric site and its GDP-mimetic ligands, harnessing allostery may provide more options for further anti-K-Ras^{G12C} cancer drug discovery because it evades the competition with the high cellular concentration of GTP/GDP and possesses more feasibility for the future design and optimization of the corresponding ligands.

3. Allosteric K-Ras^{G12C} covalent inhibitors

3.1. 6H05 series

The first salvo of K-Ras^{G12C}-specific inhibitors was reported by Ostrem et al., which is possibly the starting point for all the following K-Ras G12C mutant-specific pharmaceutical research (Ostrem et al., 2013). Using intact protein mass spectrometry (Burlingame, Tom, & Renslo, 2011; Sadowsky et al., 2011), "tethering" disulfide fragment-based screening (Erlanson et al., 2000) was carried out with 480 tethering compounds, and fragments 6H05 (**1**) (94 ± 1% (percentage modification, mean ± s.d.)) and 2E07 (**2**) (84.6 ± 0.3%) (Fig. 2A) displayed the greatest degree of modification towards C12 in mutant K-Ras. Structure-activity relationships (SARs) of **1** were investigated, and further optimization identified two potent hits, compounds **6**-^{Ostrem} (**3**) and **12**^{Ostrem} (**4**) (referred to as the 6H05 series in our article) (Fig. 2B). A crystallographic study showed that both of them covalently attached to the C12 thiol group and did not occupy the nucleotide pocket (Fig. 2C). Instead, the compounds extended into an adjacent pocket largely formed by SW2 (Fig. 2D), indicating their allosteric nature. This novel binding site was thereafter referred to as S-IIP and became a foothold for further drug design. The crystal structures revealed that S-IIP situated between the central β-sheet and SW2 of Ras (Fig. 2D) and was formed through the reordering of SW2 induced by ligand binding. In addition to the disulfide linkage, S-IIP made direct contact with the inhibitors. The dichlorophenyl group in **3** formed several hydrophobic interactions with the adjacent residues, and it was hydrogen bonded directly to Q99 and G60 (Fig. 2E). Due to these ligand-protein interactions, conformations of residues critical to GTP binding, such as Y32, G60 and T35 (Pai et al., 1989), were pronouncedly affected, and the loading of the GTP substrate was disrupted (Fig. 2F). Hence, these compounds inhibit G12C mutant K-Ras allosterically through disruption of the GTP-bound active state conformation, subverting the native nucleotide preference to favour GDP over GTP. Furthermore, biochemical and cellular experiments showed that important PPIs between Ras and its effectors such as Raf were also compromised in the presence of inhibitors due to the structural alterations of the SW2 domain, therefore blocking oncogenic Ras signalling. *In vivo* tests found that **4** could increase apoptosis and decrease viability with genotype-specificity in a K-Ras (G12C) cellular model with a half-maximum effective concentration (EC_{50}) of 0.32 ± 0.01 μM. Hence, all the results described here provide a proof-of-concept of the first successful attempt towards K-Ras^{G12C}-specific covalent inhibitor development.

3.2. Quinazoline series

S-IIP has received intense research interest from the pharmaceutical industry after its discovery. Araxes Pharma developed several patented compounds targeting it, based on which Zeng et al. carried out further structure-based optimization (Zeng et al., 2017). Starting from several quinazoline binder examples reported by Araxes Pharma, a set of analogues were profiled for their SARs. A study comparing compound **1**^{Zeng} (**5**) (Fig. 3) and its analogue with 2-amino amide substituent, **1_AM** (**6**) (Fig. 3), found that attachment of the amide group improved the compound's labelling kinetics and efficiency. Additionally, compared with its parent core, **6** could decrease the level of GTP-bound active K-Ras by approximately 80% and could inhibit the downstream phosphorylation of ERK more effectively. These results indicated that the addition of an amide substituent conferred efficiency and potency advantages. Further modification and SARs based on **6** retrieved a series of potent hits, **2_AM** (**7**), **3_AM** (**8**), and **4_AM** (**9**) (Fig. 3). The (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay (Roche) revealed that the anti-proliferative half-maximum effective concentration (IC_{50}) for these four ligands were all under the 3 μM level (2.07 μM for **6**, 2.98 μM for **7**, 1.70 μM for **8**, and 0.73 μM for **9**), which exhibited promising prospects as potential drug candidates. In addition to increased anti-proliferative potency and apoptosis-inducing ability, the selectivity for G12C-mutated K-Ras cancer cell lines was also enhanced by amide group modification. To unravel the in-depth mechanisms for their improved performances, structures of K-Ras^{G12C} in complex with **6** and **8** were solved (Fig. 4). Co-crystal structures showed that quinazoline compounds presented chemical diversity to sub-regions of S-IIP, and new conformations of S-IIP were uncovered upon their binding (Fig. 4A). X-ray structures revealed that both ligands were positioned between the α2- and α3-helices, similar to the classic S-IIP (Fig. 4B). However, substantial differences were observed for the

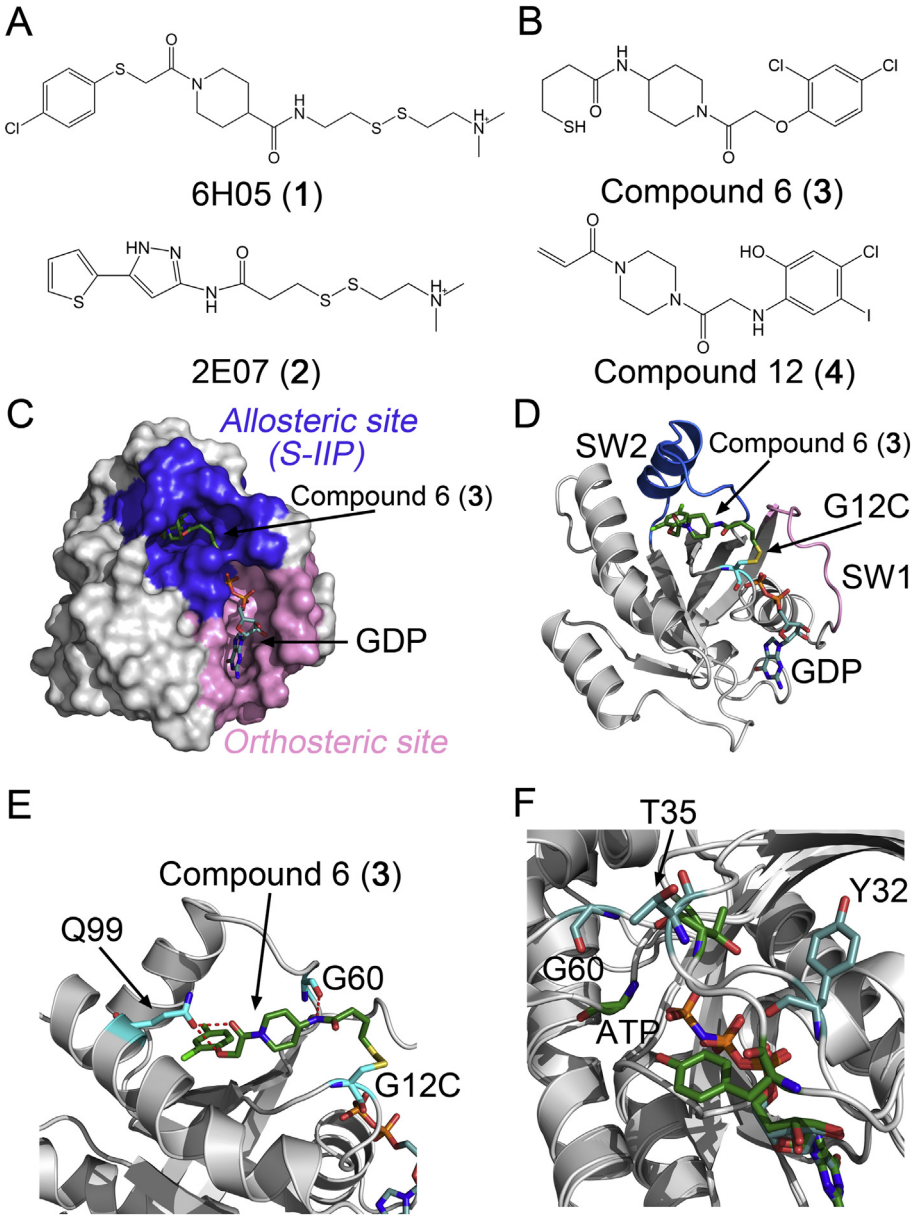


Fig. 2. (A) Chemical structures of fragments 6H05 (**1**) and 2E07 (**2**). (B) Chemical structures of covalent allosteric K-Ras^{G12C} inhibitors compounds **6**^{Ostrem} (**3**) and **12**^{Ostrem} (**4**). (C) Structural overview of the allosteric pocket for **3** on K-Ras (PDB ID: 4LUC). (D) The binding site for **3** situates between the central β-sheet and SW2 in K-Ras. (E) Interaction details between **3** and K-Ras, with hydrogen bonds highlighted by red dashed lines. (F) Conformational changes in residues critical to GTP binding induced by allosteric inhibitor binding. (Residues from ATP-bound K-Ras are shown in green, PDB ID: 4L9W; residues from **3**-bound K-Ras are shown in blue, PDB ID: 4LUC).

conformations of SW2 (Fig. 4C). In the **6**-bound K-Ras^{G12C} structure, the added amide substituent in the compound formed π-π stacking interactions with H95. The phenyl ring of the ligand caused the rotation of M72 away from the α3-helix, which displaced the α2-helix outwards, causing SW2 to shift away from the protein body in an extended form (Fig. 4D). For the **8**-bound complex (Fig. 4E), SW2 took on a different conformation upon binding (Fig. 4F). The ligand was hydrogen bonded to E63 and D69, and M72 was shifted towards the α3-helix to accommodate the naphthalene ring in the molecule (Fig. 4E). Therefore, SW2 was pinned against S-IIP in a relatively closed conformation (Fig. 4F). The additional interactions between compounds and K-Ras due to the attachment of the amide group might account for the efficiency and efficacy advantage of these molecules. Therefore, this study provided a novel strategy for future drug discovery targeting the K-Ras G12C mutant with improved pharmacological properties and selectivity.

3.3. ARS series

3.3.1. ARS-107 and ARS-853

The discovery of S-IIP on K-Ras (Ostrem et al., 2013) represents a significant step towards taming the "undruggable" Ras for cancer therapeutic interventions, and on this basis, Patricelli et al. followed a similar stream and optimized previously reported inhibitors (Patricelli et al., 2016). To enhance the compounds' cellular efficacy, iterative structure-based design was carried out, and the resulting molecules were tested both *in vitro* and *in vivo*. A strong biochemical hit, ARS-107 (**10**) (H358 cellular engagement IC_{50} = 63 μM) (Fig. 5A), was first identified, and further SAR analyses led to a more potent candidate, ARS-853 (**11**) (Fig. 5A) (IC_{50} = 1.6 μM). The crystal structure of ligand-bound K-Ras^{G12C} confirmed the covalent modification of on C12 in S-IIP by **11** (Fig. 5B). Compared with the compounds in the 6H05 series reported by Ostrem et al. (2013), **11** induced

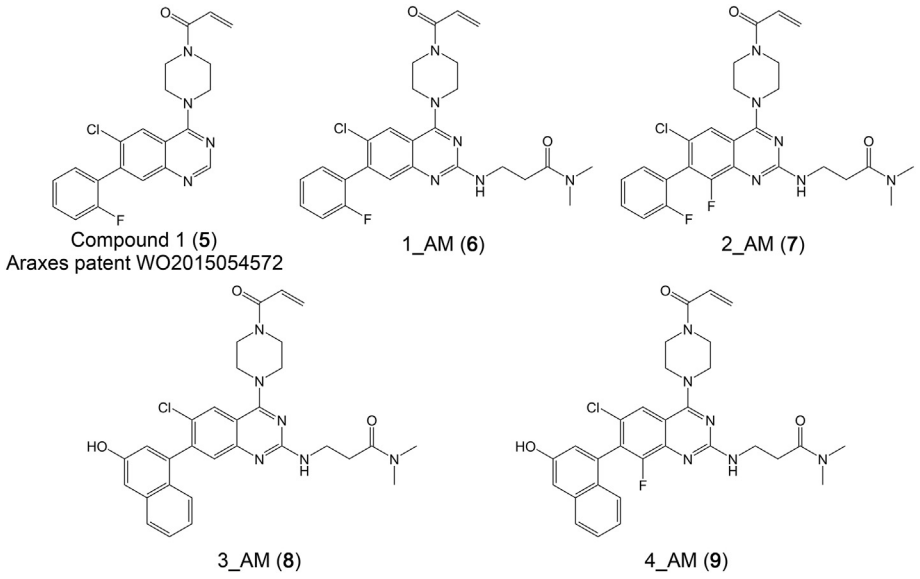


Fig. 3. Chemical structures of covalent allosteric K-Ras^{G12C} inhibitors, compound **1**^{Zeng} (**5**), **1_AM** (**6**), **2_AM** (**7**), **3_AM** (**8**), and **4_AM** (**9**).

conformational alterations in SW2 as well as S-IIP, and the binding caused a shift in M72 and rotation of the α2-helix (Fig. 5C). **11** formed hydrogen bonds with K16, R68, and D69 in K-Ras (Fig. 5D). The chloro and methylcyclopropyl moieties within it were engaged in hydrophobic interactions with M72 in K-Ras (Fig. 5D). This molecule also disrupted the PPI between K-Ras and SOS, which catalyses nucleotide exchange. Hence, **11** functioned through disruption of the critical nucleotide transition, either by intrinsic or catalysed mechanisms. Cellular experiments demonstrated a broad spectrum activity of **11** towards multiple cancer cell lines, where it increased apoptosis and decreased cell growth in both 2D and 3D assays. Furthermore, its selectivity across other free cysteines within the proteome was assessed, which showed that K-Ras^{G12C} was the primary and most effective target. Moreover, a biochemical study revealed the enhanced efficacy of **11** compared with **4**. **11** engaged the mutant cysteine with a rate constant of 76 M^{-1} s^{-1}, exhibiting a >600-fold improvement. Additionally, the IC_{50} of **11** in cellular experiments (1.6 μM) was greatly improved to reach the range of a drug candidate and was much lower than that of **4** (> 100 μM). Most remarkably, **11** could only react with the GDP-bound inactive K-Ras^{G12C} but not the GTP-bound active one, which suggested that the mutant oncoprotein cycled rapidly between GTP-/GDP-bound states rather than being trapped in an abnormally active form.

The underlying mechanisms of **11** were further studied in more detail by Lito et al. (2016). Using differential scanning fluorimetry, researchers first demonstrated the preference of **11** to bind towards GDP-bound K-Ras. Then, through experiments with mutant cells harbouring mutations that affect the guanine nucleotide cycle of Ras in cancer patients, they found that the inhibition of **11** required the GTPase activity of K-Ras and that the sensitivity of K-Ras towards **11** was decreased by the potentiation of nucleotide exchange. Co-immunoprecipitation assays showed that the PPI between K-Ras and SOS was compromised upon treatment with **11**. Hence, **11** is proposed to function by lowering the affinity of K-Ras towards nucleotide exchange factors such as SOS and disrupting the related PPIs, thereby trapping K-Ras in an inactivated form and inhibiting the related oncogenic signalling. This compound was further revealed to be a potentially robust tool compound through both computational and experimental assessments. Sautier et al. revealed that **11** approached the stringent criteria from the Structural Genomics Consortium for an *in vitro* probe, such as an on-target effect in cells <1 μM and selectivity >30-fold compared with the target protein family (Arrowsmith et al., 2015; Sautier et al., 2016). Therefore, the aforementioned studies not only provide an optimized K-Ras^{G12C}-specific allosteric inhibitor but also revolutionized our previous view that the mutant K-Ras was locked in a constitutively active form, supplying in-depth clues for future relevant drug development.

3.3.2. ARS-1620

Despite the development of numerous K-Ras^{G12C} covalent inhibitors, due to their inherent restrictions in pharmacological properties and limitations in chemical and metabolic stability, few of them have been translated into *in vivo* studies, especially in tumour models. Hence, based on the SARs for the previously reported ARS series (Patricelli et al., 2016) and the related crystallographic results (Lito et al., 2016; Patricelli et al., 2016), Li et al. shortened the flexible 2-amino-1-(piperazin-1-yl)ethan-1-one linker within **11** and its analogues and replaced it with a more rigid bicyclic scaffold, which identified a versatile lead scaffold (**12**) (Fig. 6A). Modification of the parent core in an attempt to overcome previous limitations and improve drug-like properties led to the development of a series of patented compounds (WO2015054572). Wijeratne et al. first retrieved the inhibitor compound **1**^{Wijeratne} (**13**) (Fig. 6A) from this patent and profiled its cellular activity against a series of K-Ras G12C cancer cell lines (Wijeratne et al., 2018). In cell viability assays, the IC_{50} values for **13** towards the H358, H23, H2030 and MiaPaca-2 cell lines were 0.64 μM, 0.70 μM, 0.16 μM, and 0.12 μM, respectively. Subsequently, the potential off-target effects of the compound were tested in the overall proteome consisting of over 3200 cysteine residues, and it was found that **13** exhibited high selectivity for K-Ras G12C engagement, which demonstrated its positive pharmacological and toxicological properties.

A more in-depth study was further carried out for the enantiomer of **13**, *i.e.*, ARS-1620 (**14**), by Janes et al. (2018) (Fig. 6A). Biochemical assays first found that **14** could covalently modify C12 in mutant K-Ras with an observation rate of 1100 ± 200 M^{-1} s^{-1}, a 10-fold improvement relative to **11**. Kinetic analysis revealed that this enhancement was driven by K-Ras-catalysed covalent bond formation (Hansen et al., 2018). The efficacy and selectivity for K-Ras-specific inhibition were greatly improved after optimization. **14** displayed an increase of >10-fold in IC_{50} for Ras signalling inhibition (120 nM *versus* 1700 nM) and a >100-fold window of mutant selectivity within a cellular context. Cell growth assays showed that **14** suppressed cell proliferation specifically towards *K-Ras p. G12C* cell lines (H358, MIA-PaCa2, and LU65) with an IC_{50} of 150 nM while exerting relatively benign effects on other K-Ras mutant cell lines (H441, A549, and HCT116). The risks of off-target effects on other reactive cysteines within the proteome were also ruled out using an unbiased proteome-wide screen covering

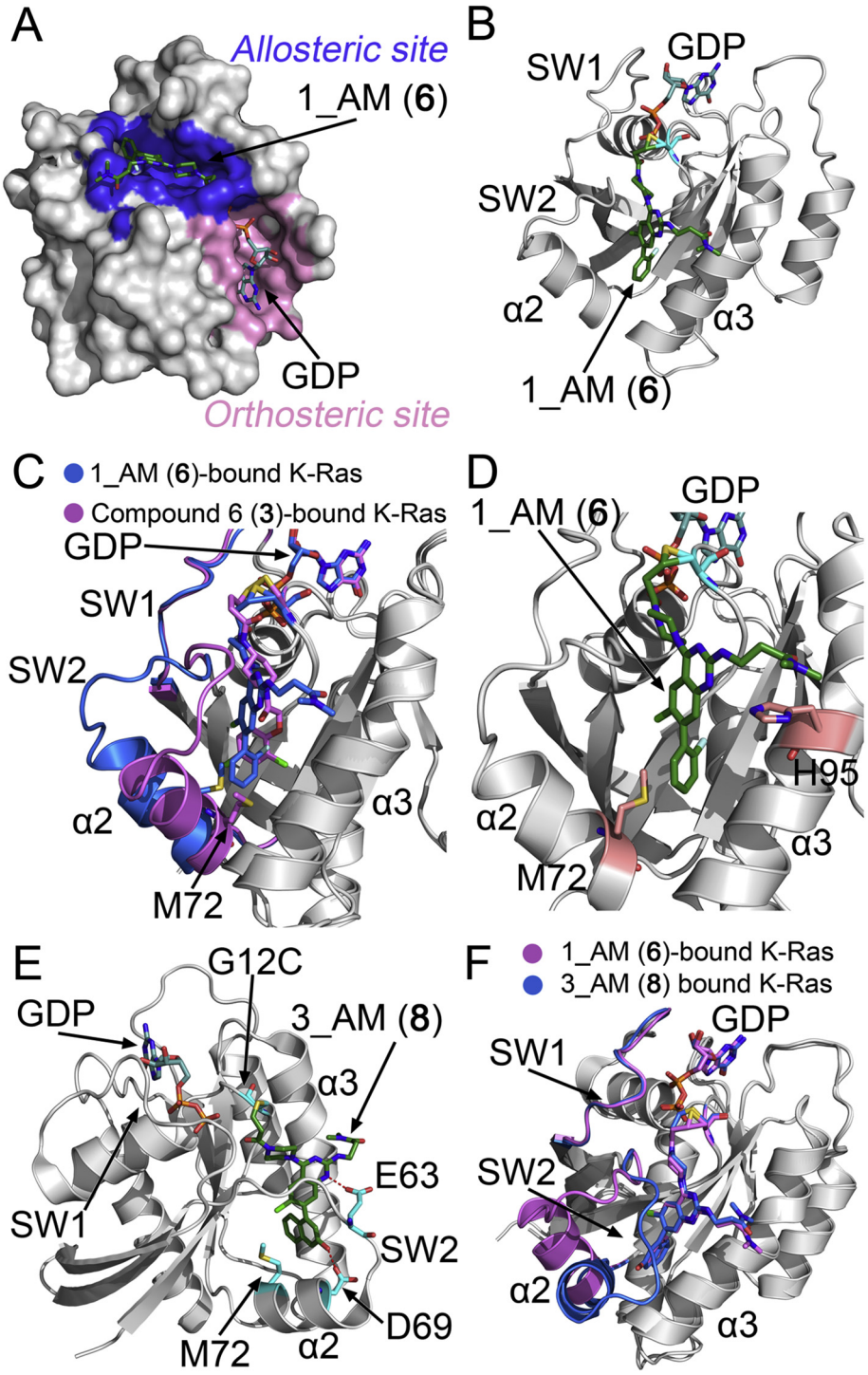


Fig. 4. (A) Structural overview of the allosteric pocket for **1_AM** (**6**) on K-Ras (PDB ID: 5V9L). (B) **6** situates in S-IIP between the α-2 and α-3 helices. (C) Structural comparison of K-Ras in complex with **6** (PDB ID: 5V9L, blue) and **3** (PDB ID: 4LUC, pink). (D) **6** forms π-π stacking interactions with H95 and displaces M72 away from the α3-helix. (E) Interaction details between **3_AM** (**8**) and K-Ras^{G12C}. (F) Structural comparison of K-Ras in complex with **6** (PDB ID: 5V9L, pink) and **8** (PDB ID: 5V0O, blue).

8501 cysteine residues across 3012 annotated proteins, which revealed that G12C in mutant K-Ras is the cysteine residue most substantially and significantly engaged by the compound. The co-crystal structure of the *holo* form of K-Ras^{G12C} was solved to provide more structural insights (Fig. 6B), which revealed a different binding mode and trajectory towards S-IIP of **14** compared with previously reported ligands (Fig. 6B, C). Compared with compounds in the ARS 853 series, **14** formed additional interactions with H95 and adopted a more rigid conformation that is more favourable for covalent binding to C12 (Fig. 6C). The fluoro group in the fluorophenol moiety extended into a hydrophobic region within S-IIP, and the hydroxyl group in the same moiety formed several water-mediated hydrogen bonds with R68, D69 and Q99 (Fig. 6D). The orientation of the fluorophenol warhead and the related intermolecular interactions might explain the distinct binding pose of **14** and its improved performance (Fig. 6C, D). Further studies in animal models first unveiled the high oral bioavailability (F > 60%) and desirable pharmacological properties of **14**. The compound also possessed sufficient blood stability for quantitative measurements *in vivo*. Experiments in both subcutaneous xenograft models and patient-derived tumour xenograft models suggested that **14** was highly selective for the

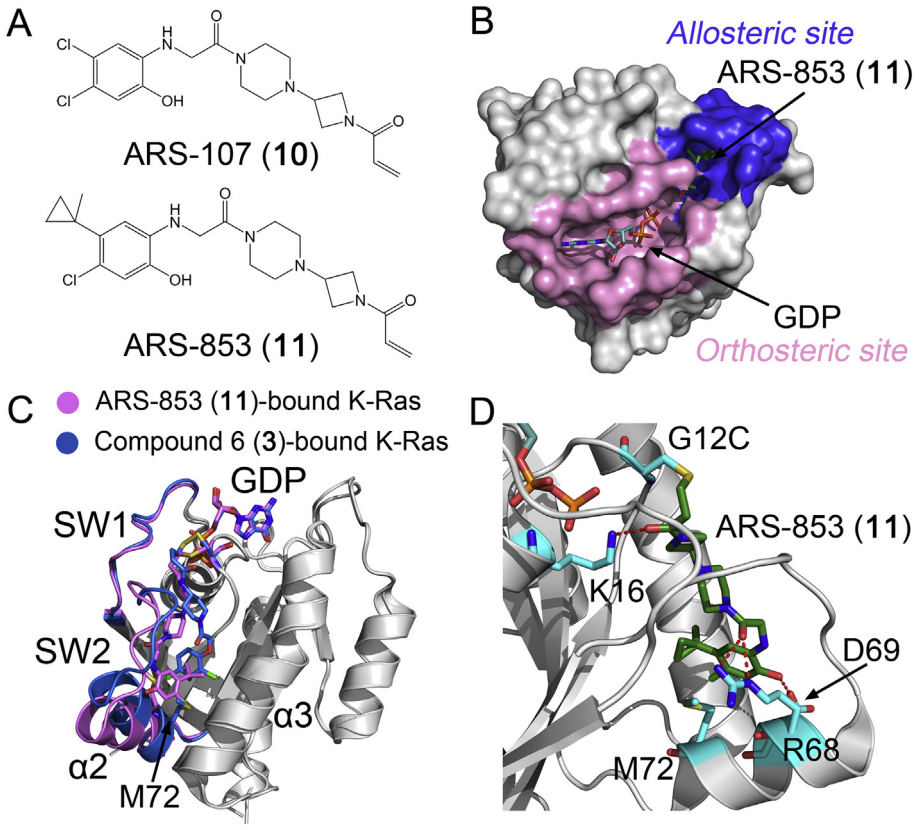


Fig. 5. (A) Chemical structures of covalent allosteric K-Ras^{G12C} inhibitors ARS-107 (**10**) and ARS-853 (**11**). (B) Structural overview of the allosteric pocket for **11** on K-Ras (PDB ID: 5F2E). (C) Structural comparison of K-Ras in complex with **11** (PDB ID: 5F2E, pink) and compound **6**^{Ostrem} (**3**) (PDB ID: 4LUC, blue). (D) Details of the interaction between **11** and K-Ras^{G12C}.

K-Ras G12C mutant and could inhibit Ras signalling, thereby effectively inducing marked tumour regression. Hence, the remarkable performances of **14** both *in vitro* and *in vivo* underscore its promising therapeutic potential for translation into future anti-tumour drugs. This compound also represents a valuable pharmacological tool for future interrogation of K-Ras biology in animal model studies.

3.4. Tetrahydropyridopyrimidines series

In addition to the ARS series, another set of K-Ras^{G12C}-specific covalent allosteric inhibitors with outstanding performances was developed through efforts from the pharmaceutical and biotech industries (Fell et al., 2018). By employing a similar workflow as that described by

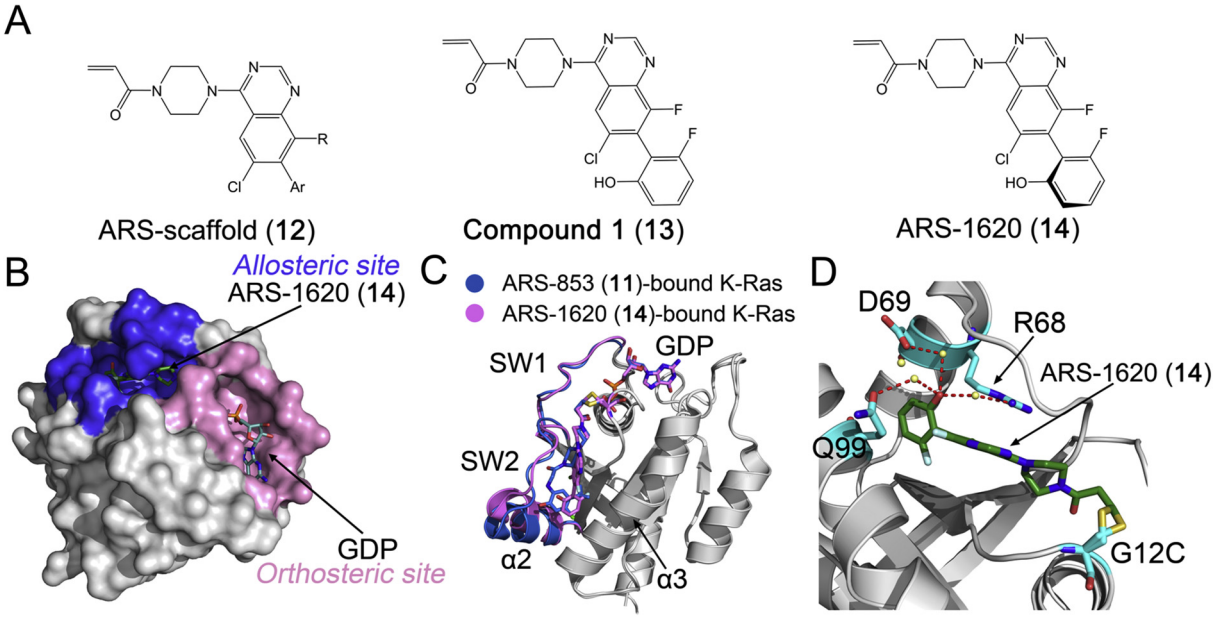


Fig. 6. (A) Chemical structures of the scaffold (**12**) of ARS-series covalent allosteric K-Ras^{G12C} inhibitors, compound **1**^{Wijeratne} (**13**) and ARS-1620 (**14**). (B) Structural overview of the allosteric pocket for **14** on K-Ras (PDB ID: 5V9U). (C) Structural comparison of K-Ras in complex with **11** (PDB ID: 5F2E, blue) and **14** (PDB ID: 5V9U, pink). (D) Interaction details between **14** and K-Ras^{G12C}.

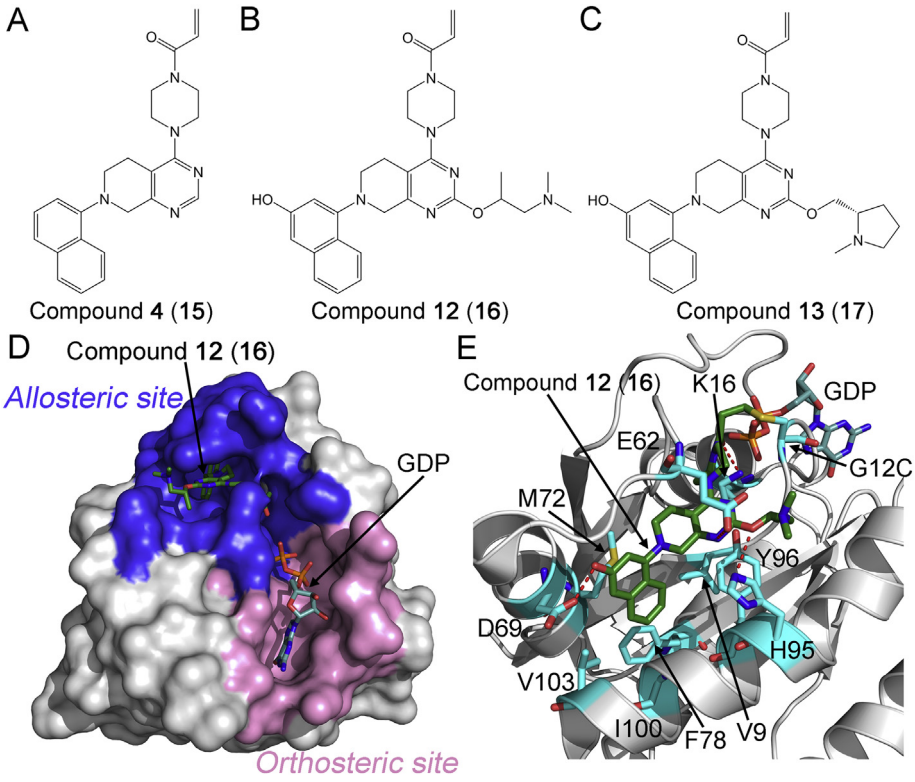


Fig. 7. (A) Chemical structure of compound $\mathbf{4}^{Fell}$ (**15**). (B) Chemical structure of compound $\mathbf{12}^{Fell}$ (**16**). (C) Chemical structure of compound $\mathbf{13}^{Fell}$ (**17**). (D) Structure overview of K-Ras^{G12C} in complex with covalent allosteric inhibitor **16**. (PDB ID: 6N2K) (E) Details of the interaction between covalent allosteric inhibitor **16** and G12C K-Ras. Residues critical for the binding of **16** are highlighted as sticks and shown in cyan, and hydrogen bonds are displayed as red dashed lines.

Ostrem et al. (2013), Fell et al. screened the Array BioPharma covalent fragment collection against G12C K-Ras with a protein modification assay. Screening hit elaboration in an iterative library format and structure-based drug design gave compound $\mathbf{4}^{Fell}$ (**15**) (Fig. 7A). In MS-based assays, **15** exhibited a covalent modification rate of 13% towards K-Ras^{G12C}, and it was then exploited as the parent core for further optimization. A series of analogues of **15** were synthesized to explore its SARs. In an attempt to induce more interactions between the compound and K-Ras, a hydroxyl group was added to the naphthalene group of **15** to enable potential hydrogen bonding, and C-2 substitution in the pyrimidine ring was introduced. These modifications retrieved two enhanced hits, compounds $\mathbf{12}^{Fell}$ (**16**) (Fig. 7B) and $\mathbf{13}^{Fell}$ (**17**) (Fig. 7C). Compared with **15**, **16** and **17** showed improved modification rates (21% and 84%, respectively), and they could inhibit K-Ras signalling and suppress downstream ERK phosphorylation in the H358 G12C K-Ras-driven cancer cell line with nanomolar IC_{50} values (540 nM and 70 nM, respectively). Hence, they represented advanced leads for mutant-specific inhibition of G12C K-Ras. Their covalent allosteric binding modes were further ascertained through a crystallographic study (Fig. 7D). Within the co-crystal, the naphthyl ring of the ligand formed hydrophobic interactions within the pocket consisting of V9, M72, F78, Y96, I100, and V103. Hydrogen bonds were formed between **16** and K16, D69, and H95. From the C-2 amino substituent, the inhibitor also made salt-bridge interactions with E62 and cation-π interactions with H95 (Fig. 7E). Through these interactions, **16** captured K-Ras^{G12C} in a GDP-bound inactive conformation, thereby inhibiting the related oncogenic signalling.

This series of compounds were then advanced to *in vivo* testing after demonstration of their selectivity in three non-G12C mutant cell lines. ADME (Absorption, Distribution, Metabolism, and Excretion) and pharmacokinetic property evaluations verified **17** as a viable candidate for tumour model studies, and intraperitoneal (IP) injections of 30 or 100 mg/kg were identified as ideal means of animal administration, which could ensure that the drug plasma concentration covered the free-fraction-adjusted cellular IC_{50} for at least 1–3 h after administration. In MIA PaCa-2 xenograft models, IP QD (*quaque die*, once a day) treatments for 5 days could trigger rapid tumour regression, and in the best cases, after 25 days of administration, the xenograft tumour could be completely eliminated. Furthermore, LC-MS (liquid chromatography-mass spectrometry)-based assays showed that K-Ras^{G12C} engagement in tumour tissues by **17** could be maintained for >65% after dosing, suggesting the high on-target efficiency and sustained binding of **17**. Most importantly, mouse models were tolerant towards **17**, and no side effects, such as weight loss or other adverse symptoms, were reported. Hence, the tetrahydropyridopyrimidines series, as exemplified by **17**, represents a class of advantageous K-Ras^{G12C}-specific covalent allosteric inhibitors that have positive safety profiles and that possess great potential in future anti-K-Ras drug discovery. In fact, very recently, Mirati Therapeutics, Inc. has launched one of the very first clinical trials (NCT03785249, recruiting) for G12C K-Ras-specific covalent inhibitors with MRXT849 (structure not disclosed yet) derived from the tetrahydropyridopyrimidines series. This effort again underscores that mutant-selective K-Ras covalent allosteric inhibitors will be a promising tactic for the development of therapeutic agents against Ras-driven cancers.

4. Orthosteric K-Ras^{G12C} covalent inhibitors

4.1. SML series

In addition to inhibit K-Ras allosterically through the novel S-IIP, covalent K-Ras^{G12C} binders also provide new directions for orthosteric site inhibition. The development of small-molecule inhibitors directly targeting the active site of Ras has long been hampered by the subnanomolar affinity of its substrates, GDP and GTP, and their high intracellular concentrations. In light of the recent success of covalent kinase inhibitors, which irreversibly target the ATP-binding sites and prevent ATP loading (Zhang et al., 2012; Zhou et al., 2009; Zhou et al., 2010),

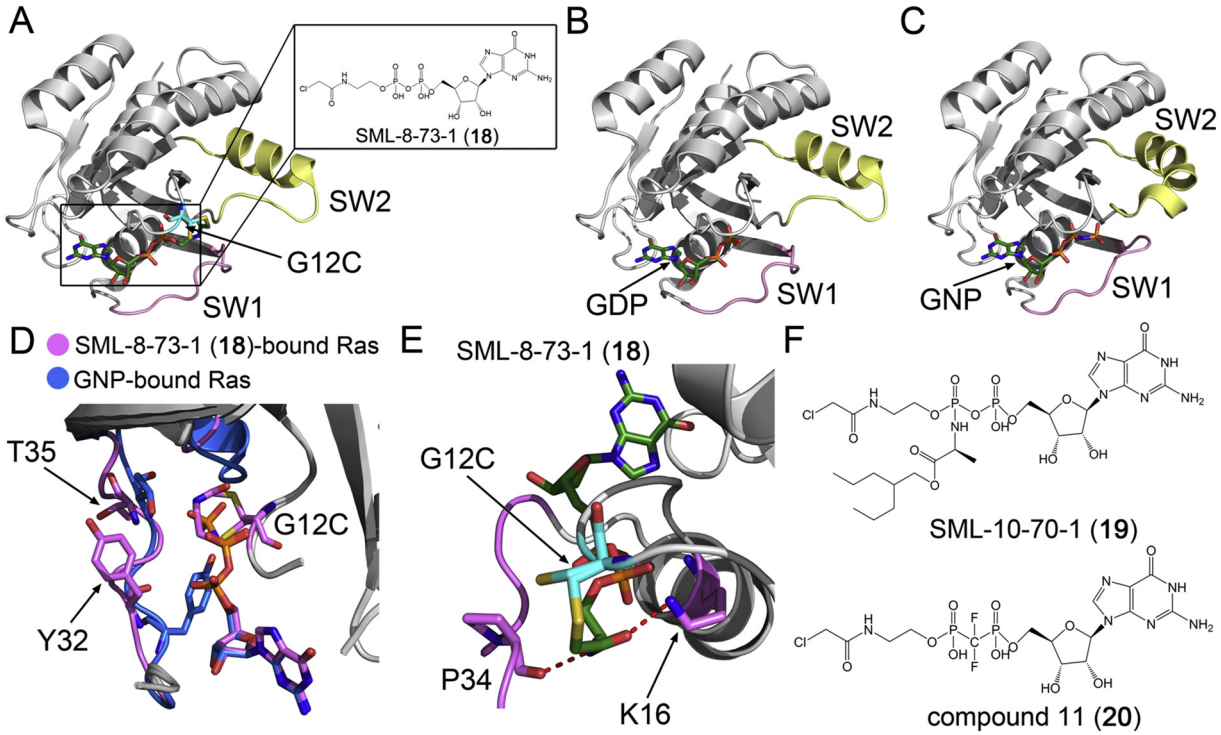


Fig. 8. (A) Structural overview of K-Ras^{G12C} in complex with covalent orthosteric inhibitor SML-8-73-1 (**18**) (PDB ID: 4NMM). (B) The conformation of SW2 in GDP-bound Ras is similar to that in **18**-bound Ras. (PDB ID: 4LDJ). (C) The conformation of SW2 in GNP-bound Ras is different from that in **18**-bound Ras (PDB ID: 4L9W). (D) Structural comparison of Ras in complex with **18** (PDB ID: 4NMM, pink) and GNP (PDB ID: 4L9W, blue). (E) Details of the interaction between **18** and K-Ras. (F) Chemical structure of covalent orthosteric K-Ras^{G12C} inhibitor SML-10-70-1 (**19**) and compound **11**^{Xiong} (**20**).

covalent modifiers may provide possible fashions to tackle the high competing nucleotide concentration problem in drugging Ras orthosteric site.

Starting from the GDP scaffold, Lim SM. et al. designed the first series of covalent substrate-competitive inhibitors targeting the catalytic site by modifying diphosphate compounds with various electrophiles and different linker lengths between the electrophile and β-phosphate (Lim et al., 2014). SML-8-73-1 (**18**) (Fig. 8A) was identified as the leading candidate, and its G12C-specific addition was first demonstrated with electrospray ionization mass spectrometry. Proteolytic digestion of K-Ras^{G12C} and analysis of the resulting peptides by nano-LC/MS also confirmed the mutant-specific covalent binding manner. The binding efficiency of **18** was tested in the presence of 1 mM GDP or GTP, simulating cellular conditions, and the results showed that after 2 h incubation, **18** could still bind to >95% K-Ras G12C in competition with the high-affinity guanine nucleotide substrates. Hydrogen exchange (X) mass spectrometry (MS) revealed that **18** altered the conformation of the nucleotide binding pocket, inducing it similar to the GDP-bound form, which suggested that **18** stabilized the inactive state of K-Ras^{G12C}. More structural details were unveiled by crystallography (Hunter et al., 2014), which revealed that in the **18**-bound structure, both SW1 and SW2 adopted inactive and open conformations (Fig. 8A–C). Y32 and T35, which projected towards the active site in the GTP-bound active form, rotated away upon compound binding (Fig. 8D). Moreover, **18** made hydrogen bonds with K16 and P34, which partly explained its structural effects (Fig. 8E). Since the function of Ras mainly depends on its PPIs mediated through SW1 and SW2 with effector partners such as Raf, fluorescence polarization assays and a functional biochemical assay using AlphaScreen (PerkinElmer) technology were carried out to measure the binding event of Raf towards Ras. The results showed that the PPI between Ras and Raf was compromised by **18** because it rendered Ras in a GDP-bound-like inactive state. Additionally, PPIs from other effectors downstream to Ras, such as PI3K, were also disrupted by **18** due to the steric clashes induced by inhibitor binding. Taken together, **18** was demonstrated to be an effective orthosteric inhibitor for oncogenic K-Ras G12C mutant function in a substrate-competitive manner.

Nevertheless, **18** contains two negatively charged phosphate groups, which leads to difficulty in membrane penetration. “Caging” is a common strategy where charged ions are chemically modified to mask the charged moieties, thereby enabling passive cellular uptake (Adams & Tsien, 1993). Based on this methodology, SM-10-70-1 (**19**), an optimized and partially caged version of **18**, was synthesized (Fig. 8F). **19** exhibited enhanced cell permeability and competitively inhibited K-Ras^{G12C} through covalent binding. K-Ras-dependent signalling, such as the Akt and ERK pathways, was also suppressed. Moreover, the antiproliferative activity of **19** was demonstrated in several cancer cell lines expressing the K-Ras G12C mutation. However, its efficiency and target selectivity still need further improvement. In addition, “caging” compounds frequently suffer from instability; therefore, another round of SARs was investigated, which retrieved the promising compound **11**^{Xiong} (**20**) (Fig. 8F). By substituting the central oxygen in the phosphate anhydride bond in **18** and **19** with a methylene group, **20** was greatly improved in its chemical and enzymatic stability. Despite its 40-fold reduced affinity compared with **18**, **20** might still be a more favourable and facile prodrug candidate.

5. Conclusions and perspectives

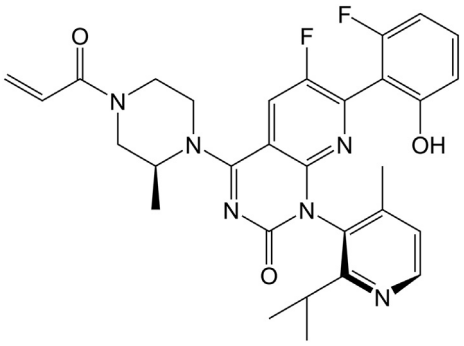
As one of the most common genetic lesions in human malignancy, Ras mutations appear in >90% of pancreatic, 45% of colon and 35% of lung cancers, and the resulting abnormally activated Ras GTPase acts as an oncogenic driver for tumorigenesis and tumour progression (Cox & Der, 2010; Eser et al., 2014; Holderfield et al., 2014; Krens et al., 2010; Lu, Jang, Gu, et al., 2016; Lu, Jang, Muratcioglu, et al., 2016; McCormick, 2015a, 2015b; Prior et al., 2012). Ras has been notorious for its smooth protein surface, high substrate affinity, and lack of evident binding pockets, and its inhibition has long remained one of

the most prominent unsolved conundrums in oncology drug development (Cox et al., 2014; Lu, Jang, Gu, et al., 2016; Lu, Jang, Muratcioglu, et al., 2016; Papke & Der, 2017). Recently, the US National Cancer Institute launched the Ras Initiative, a US$10 million per year intramural effort to find new viable approaches to attack Ras-driven cancers (Esposito, Stephen, Turbyville, & Holderfield, 2018; Ledford, 2015). This effort unites academia and the pharmaceutical industry and fuels more momentum in the renaissance of Ras study and related therapeutic agent discovery. One of the key breakthroughs in attempts to drug the "undruggable" Ras is tailoring G12C K-Ras through cysteine-binding covalent inhibitors, which offer a promising opportunity to modulate one of the major oncogenic Ras mutants with improved efficacy and selectivity (Janes et al., 2018; Lim et al., 2014; Ostrem et al., 2013; Patricelli et al., 2016; Wijeratne et al., 2018; Xiong et al., 2017; Zeng et al., 2017). This novel methodology not only supplies valuable pharmacological tools to interrogate K-Ras biology but also contributes to bringing the historically "undruggable" Ras into the clinic.

Recent progress in K-Ras modulator development has retrieved a series of G12C mutant-specific covalent inhibitors acting through either allosteric or orthosteric mechanisms (Fell et al., 2018; Janes et al., 2018; Lim et al., 2014; Ostrem et al., 2013; Patricelli et al., 2016; Wijeratne et al., 2018; Xiong et al., 2017; Zeng et al., 2017), and in general, allosteric inhibitors possess greater potential for future drug discovery. Based on biochemical assays, allosteric inhibitors exhibit higher efficacy, and the best of these compounds, such as **14** and **17**, are highly efficacious towards multiple cancer cell lines and even in xenograft mouse models (Fell et al., 2018; Janes et al., 2018). For the orthosteric ligands, their difficulties mainly stem from the competition with the high-affinity guanosine nucleotide substrates (Lu, Jang, Gu, et al., 2016; Lu, Jang, Muratcioglu, et al., 2016; Wilson & Tolias, 2016). Moreover, structural analysis of their binding pockets reveals that the allosteric sites can provide more possibility for future drug discovery and optimization. The allosteric S-IIP, first identified by Ostrem et al. and later exploited by almost all covalent allosteric ligands, is a highly elastic pocket (Ostrem et al., 2013), which may be explained by the dynamic nature of the Ras protein. Structural comparison finds that this inducible cavity is only fully formed upon compound loading and that different ligands adopt different binding poses within it. Ostrem et al. found that, through medicinal chemistry efforts, S-IIP can be expanded to accommodate larger and different compounds (Ostrem & Shokat, 2016), which underscores the great potential of this allosteric pocket in future anti-Ras therapeutic agent design.

Ras^{G12C} covalent inhibitors display pharmacological merits such as enhanced potency and prolonged drug action compared with noncovalent ligands, and they represent the state-of-the-art breakthrough in the attempt to drug the historically "undruggable" Ras. Nevertheless, this class of inhibitors still suffers from adverse events of covalent modifiers called idiosyncratic adverse drug reactions (IADRs) resulting from off-target effects, chemically reactive drug metabolites, and immunogenic covalent adducts (De Cesco, Kurian, Dufresne, Mittermaier, & Moitessier, 2017; Gonzalez-Bello, 2016; Kalgutkar & Dalvie, 2012; Mah, Thomas, & Shafer, 2014; Singh, Petter, Baillie, & Whitty, 2011). Hence, intensive efforts are still needed in this field for further drug optimization and development.

One of the most widely-used approaches for inhibitor discovery is large-scale screening through allele-specific cell lines. However, we notice that even though all harbouring $K\text{-}Ras^{G12C}$ mutation, cancer cell lines used in previous studies such as H358, MIA-PACA2, *etc.* actually display differential sensitivities towards the same inhibitor compound. Similar phenomena were also observed for tumour cells cultured under 2D or 3D conditions. Such findings may be attributed to the varied genetic background and the diversified environmental conditions of the corresponding cancer cells. Therefore, in future related drug development, caution should be paid towards results from cellular experiments. Another important technological progress in the discovery of K-Ras G12C inhibitors is the application of bioinformatics tools. In the study carried out by Nnadi et al., *in silico* covalent docking was combined with hydrogen-deuterium exchange mass spectrometry, which retrieved a series of hit compounds with favourable potency (Nnadi et al., 2018). This success highlighted the efficiency of computational algorithms in hit discovery and lead identification, and the incorporation of both bioinformatics and experimental methodologies will surely accelerate the pace of future K-Ras covalent drug design.



AMG 510 (**21**)
Amgen patent US20180177767

Fig. 9. Chemical structure of AMG 510 (**21**) from Amgen Inc..

Additionally, previous research found that K-Ras covalent inhibitors favour binding the inactive protein, and specifically, the allosteric S-IIP predominantly exists in the GDP-bound topology of Ras (Lito et al., 2016). However, since $K\text{-}Ras^{G12C}$ is not locked in a constitutively active state but still undergoes nucleotide flux between active and inactive conformations, this dynamic process may also provide an emerging opportunity for inhibitor design. The recent advances in crystallography and structural bioinformatics studies have unravelled several intermediate states along the (de)activation pathway of Ras (Chakrabarti, Jang, & Nussinov, 2016; Geyer et al., 1996; Hall, Bar-Sagi, & Nassar, 2002; Lu, Jang, Muratcioglu, et al., 2016; Ma & Karplus, 1997; Muraoka et al., 2012; Ni, Song, Zhang, & Lu, 2017; Spoerner, Nuehs, Herrmann, Steiner, & Kalbitzer, 2007). Our better understanding of the Ras structural dynamics and conformational ensembles will be instructive to the structure-based covalent drug design in the future (Cox et al., 2014; Lu, He, Ni, & Zhang, 2019; Lu, Jang, Gu, et al., 2016; Lu, Ji, Ni, & Zhang, 2018; Lu, Shen, & Zhang, 2019; Papke & Der, 2017).

Furthermore, advanced analytical techniques can help avoid or at least attenuate the unwanted side effects of covalent ligands, such as the LC-MS/MS methodology and fluorescence or radiochemical detection techniques to identify chemically reactive drug metabolites (Thompson, Isin, Ogese, Mettetal, & Williams, 2016) and chemoproteomic techniques for total proteomic cysteines screening and demonstration of binding selectivity towards the G12C K-Ras target (Janes et al., 2018; Wang, Weerapana, Blewett, & Cravatt, 2014). Last but not the least, the therapeutic value of $K\text{-}Ras^{G12C}$ covalent inhibitors can also be significantly enhanced by the latest concept of combination therapy. According to works by Patricelli et al., co-administration of ARS-853 and RTK or EGFR inhibitors could achieve better inhibition towards the Ras-related oncogenic signals (Patricelli et al., 2016). Hence, we believe that by mitigation of the upstream activation signalling, we can maximize the therapeutic potential of the $K\text{-}Ras^{G12C}$ covalent inhibitors through combination therapies.

In summary, although the path towards overcoming the "undruggability" of Ras is tortuous due to the great challenges posed by the difficult structure and topology of Ras protein, the $K\text{-}Ras^{G12C}$-specific covalent inhibitors are still established as an exciting and promising direction in the development of effective therapeutic strategy for Ras-driven cancers. Very recently, two K-Ras G12C-specific inhibitors, MRTX849 (Mirati Therapeutics Inc., NCT03785249) (structure not disclosed yet) and AMG 510 (**21**) (Amgen Inc., NCT03600883) (Fig. 9),

have reached clinical evaluation by the US Food and Drug Administration (FDA). The former, derived from the tetrahydropyridopyrimidines series, was revealed as a covalent allosteric inhibitor targeting S-IIP, which again highlights the great promise of this strategy. In principle, the covalent binder approach can be further exploited to achieve specificity for the inhibition of other important Ras mutants, such as G12D or G13D (Ostrem & Shokat, 2016). Additionally, it may be applicable to some previously "intractable" targets in cancers, especially those harbouring acquired oncogenic cysteine mutations such as FGFR3 and TP53 (Visscher et al., 2016). In the future, we hold cautious optimisms that the covalent mutant-specific inhibitors will become an up-and-coming trend in anti-Ras cancer therapeutics development and make "drugging Ras", a historical "Mission Impossible", become possible.

Declrations of Competing Interest

The authors declare that there are no conflicts of interest.

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