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Review article

Mechanistic Insight on Ras Inhibition Strategies in Cancer Therapy

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Abstract

Ras proteins are considered as one of the most critical cancer initiators. Mutations of this protein family lead to the continuous activation of the proliferation pathways. Therefore, many efforts have been taken to design the anti-mutant Ras drug candidates. Regardless of the development of promising inhibitors of Ras G12C mutant in a specific cancer type, there is no approved inhibitor of Ras mutants in the clinic. One of the significant limitations is to inhibit particular mutants and not to affect the wild-type Ras variants. Here we present a review on the mechanism of action of the Ras proteins to get a better insight into the strategies utilized to inhibit Ras-mutated cancers. The direct Ras inhibition strategies are then highlighted to obtain a better perspective of possible promising approaches to target Ras proteins in cancer therapy.

Keywords: Ras, Drug development, Cancer therapy, Structural drug design

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1. Introduction

Cancer is known as a condition characterized by the lethal, uncontrolled, and rapid growth of cells. This disease is one of the major concerns worldwide due to the lack of efficient treatments, despite the efforts made to discover new chemotherapeutic agents.[1] Based on the reports, cancer is considered the second cause of death worldwide.[2] Consequently, it is vital to investigate new cancer therapeutic agents with selective properties on cancer cells. Identification of cell-cycle regulators and apoptosis pathways represents a perspective for discovering and developing new potential antitumor agents.[3]

Kinases are essential parts of cellular pathways, and the dysfunction of these molecules can cause different disorders.[4] The Ras kinase is one of the most well-known kinases involved in regulating cell growth and proliferation. Mutations of Ras can induce uncontrolled cell growth due to the permanent activation of downstream pathways.[5] Mutations in the Ras family drive 30 percent of all human cancers.[6] The most effective Ras mutations occur in positions 12, 13, and 61, which defects both GTPase activity and protein-dependent activation of Ras. Therefore, the increased concentration of Ras protein's active form (bound to GTP) leads to augmented cellular proliferation.[7]

Accordingly, Ras, and specifically K-Ras, as the most frequent cancer-driven Ras isoform, is a promising target for cancer therapeutic design. However, no effective Ras inhibitor is in the market so far.[8] Some reasons for K-Ras targeting difficulty include a) no druggable pocket on K-Ras protein to design conventional small molecule drug b) no confirmed allosteric regulatory site on K-Ras protein [9] c) the dynamicity of K-Ras structure in active and inactive forms d) the picomolar affinity of GDP/GTP K-Ras makes it rather impossible to be inhibited by a small molecule.[10]

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Various approaches have been taken in recent years, and some successes in K-Ras targeting were achieved. In this review, the characteristics of the Ras family and K-Ras isoform specifically are described. Additionally, the strategies of direct K-Ras inhibition and the structural challenges and limitations in this regard are described. Finally, the future perspective and promising approaches are highlighted.

2. Ras subfamily proteins

2.2 Ras protein

In the 1960s, it was observed that some viruses, called "src", cause sarcoma tumor formation in mice and rats.[11,12] Then it was found that they were regular segments of the rat gene designated "Ras" due to their ability to cause rat sarcoma. Scolnick and colleagues performed a series of studies to define the characteristics of these viral genes. Consequently, it is recognized that these viral genes have a cellular origin; they encode 21 kDa proteins that bind GDP (Guanosine diphosphate) and GTP (Guanosine triphosphate) and are connected to the plasma membrane.[13] Today, "src" is called a tyrosine kinase that inhibits Ras by phosphorylating the Tyrosine 32.[14]

Ras proteins are small, membrane-associated, that switch between active GTP bound and inactive GDP bound conformations (Figure 1).[15] Ras proteins are well-known kinases taking part in many growth signaling pathways. Notably, Ras kinase mutations can permanently activate and stimulate downstream pathways, leading to excessive cell growth and cancer.[5]

2.2.1 Ras isoforms

The human Ras genes are including H-Ras, N-Ras, and K-Ras4A and K-Ras4B proteins with a range of 188 to 189 amino acids. All these isoforms are quite similar (about 90%) in

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the G domain (residues 1–166), despite significant variations in the hypervariable region (HVR). Having Ras proteins undergone post-translational modifications, the HVR is fused to the cell membrane.[16] H-Ras, K-Ras, and N-Ras are tumor oncoproteins that belong to a larger superfamily of GDP and GTP binding proteins, which hydrolyze GTP into GDP.[17] The difference between the isoforms of K-Ras4A and K-Ras4B is the additional C-terminal 22 or 23 amino acids in K-Ras4A.[18] Despite the high similarity of different Ras proteins and especially K-Ras variants, they express individual properties. For instance, exclusively K-Ras4B, as the predominant isoform of K-Ras in human cells, can be phosphorylated at Serine 181; however, K-Ras4A is the most similar to the retroviral K-Ras.[19]

2.2.2 Mechanism of Ras function

Ras proteins are molecular binders and switches of GDP and GTP molecules. Accordingly, the consequent cellular signaling is induced by Ras-GDP switching to Ras proteins' GTP state.[20] As illustrated in Figure 1, the conversion of GDP to GTP-bound form can be accelerated by the Guanine nucleotide exchange factors (GEFs) while GTPase-activating proteins (GAPs) mediate the formation of the GDP-bound state (Figure 1).[21] Therefore, the amount of active and inactive Ras is regulated by these large, multi-domain proteins. In other words, the Ras proteins' dependence on GEFs and GAPs allows the regulatory signals to alter the Ras function to switch between on and off states.[22]

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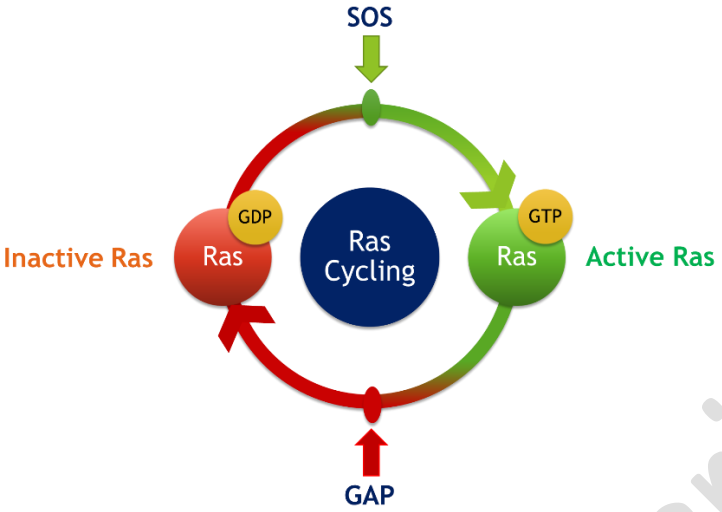

Figure 1. Mechanism of switching Ras between on and off states. Ras cycles between active GTP-bound and inactive GDP-bound states. The GEFs (such as SOS) and GAPs accelerate the formation of the active and inactive state of Ras, respectively.

GEFs, GAPs, and other effectors are activated when employed in the plasma membrane and positioned in the proximal direction relative to Ras. Appropriate positioning by recruitment to the plasma membrane increases their binding constant. Additionally, the membrane's local lipid composition may affect the binding of Ras and its effectors.[23] Ras is associated with membrane to activate downstream pathways. Cysteine residues in HVR need prenylation and palmitoylation to bind the Ras protein to the membrane. Prenylation (also known as lipidation) of Ras proteins is done on the cysteine residues of a C-terminal motif by the farnesyltransferase enzyme. Afterward, farnesylated cysteine residues are carboxymethylated by Isoprenyl Cysteine Carboxyl Methyltransferase (ICMT). The Ras protein binding to the membrane depends on the membrane's lipid composition and the electrostatic charge of C-terminal residues of HVR.[24]

Ras is often in the inactive state until signals evoke GDP/GTP exchange.[25] Binding GEFs to Ras changes the conformation of switch regions and P-loop; therefore, the GDP's affinity to

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Ras decreases. Release of GDP from Ras results in the GTP association. Due to Ras proteins' equal affinity to GDP and GTP, Ras mainly binds to GTP, which is abundant in cells (concentration ratio of GTP to GDP is about 10). Finally, GTP binding dissociates the GEF and forms the active conformation of Ras, which binds downstream effectors.[24]

Structurally speaking, the hydrogen bonds between Tyrosine 35 and Glycine 60 of Ras proteins and GTP's γ-phosphate lead to the active Ras-GTP conformation in the Switch-I and Switch-II regions, called "closed conformation". Hydrolysis of GTP diminishes the interaction of γ-phosphate with the switch regions. Therefore, the switches return to the flexible "open conformation" of Ras.[26] GTP hydrolysis happens in two ways, including the intrinsic and the GAP mediated hydrolysis. Ras proteins hydrolyze GTP intrinsically at a relatively slow rate, with approximate disassociation constant of 6×10^{-4} 1/s. GAP increases the dissociation step by several folds.[24]

2.2.3 Ras signaling pathways

Raf-MEK-ERK, MEKK-SEK-JNK, PI3K-Akt-NF/KappaB, p120/GAP-p190/B-Rac-NF/KappaB, and Raf-MEKK1-IKK-I-KappaB-NF/KappaB are the main signaling pathways known to be activated by Ras proteins. Moreover, Ras signaling contributes to numerous cellular processes, for example, cell proliferation, cell apoptosis, cell migration, and cell differentiation.[27] Besides, MAPK and PI3K pathways are crucial pathways of developing cell growth and survival, and Ras proteins are key regulatory switches of these pathways.[28]

Growth factors, receptor tyrosine kinases (RTKs), T-cell receptors (TCR), and PMA (Phorbol-12 myristate-13 acetate) are considered to be extracellular signals that activate Ras proteins.[27] For example, in a pathway, EGF (epidermal growth factor) releases from the membrane by TACE/ADAM-17 enzyme and binds to its receptor, epidermal growth factor

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receptor (EGFR), leading to EGFR's dimerization and phosphorylation. Consequently, the growth factor receptor-bound protein 2 (GRB2) and SOS proteins attach to the complex of EGF-EGFR. Therefore, SOS (in complex with GRB2) binds Ras and shows GEF activity. SOS accelerates GDP molecules dissociation from Ras proteins, and GTP molecules are allowed to bind Ras.

Following Ras activation, GTP-Ras employs the Raf proteins on the cell surface [28]. Raf binds Ras with higher affinity than other effectors or GAPs, as the range of affinity for Raf kinases is about 10–50 nM, whereas other effectors bind in the micromolar range. So GAPs cannot terminate Ras signaling to Raf. On the other hand, the recruitment of GAPs to the membrane increases their local concentration and increases the likelihood of interaction with Ras.[24] Raf protein family has three members of Serine/Threonine kinases.[27] Raf proteins' binding to active Ras alters Raf's conformation and relieves them from their inhibitors, called 14-3-3 proteins. Therefore, Raf proteins form heterodimers and bind to the KSR1 enzyme. This complex phosphorylates MEK protein leading to ERKs phosphorylation and activation. Then, ERK changes the activity status of several transcription factors in the nucleus, such as Jun and Fos. Typically, this process is terminated by Ras switching to the GDP inactive state.[28]

The other Ras main pathway is the PI3K signaling pathway. The vital cellular functions such as transcription, translation, and cell cycle are controlled by the PI3K-Akt-mTOR pathway. PI3K is a kinase family member that phosphorylates the corresponding Inositol phospholipid 3’-OH group in plasma membranes. One of the PI3K family stimulators is Ras oncogene protein. While Ras-GTP's relative affinity for PI3K is low, their spatial and mutual interactions are increased when these proteins are recruited to the plasma membrane.[24]

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The Ras and the PI3K's Ras binding domain (RBD) bind via antiparallel β-sheet interactions. Therefore, Ras binding to the RBD of membrane-anchored PI3K may lead to a PIP_2-binding-favored state of PI3K.[29] Active PI3K turns the substrate PI-4,5-P2 into the PI3,4,5-P3. At that time, the PIP3 second messengers bind to the Serine/Threonine kinases such as PDK-1 and Akt-PKB.[27] On the other hand, PIP3 dephosphorylation to PIP2 can be performed by the tumor suppressor protein phosphatase and tensin homolog (PTEN), which ends the signal.[28] The illustration of Ras-mediated cell signaling pathways is shown in Figure 2.

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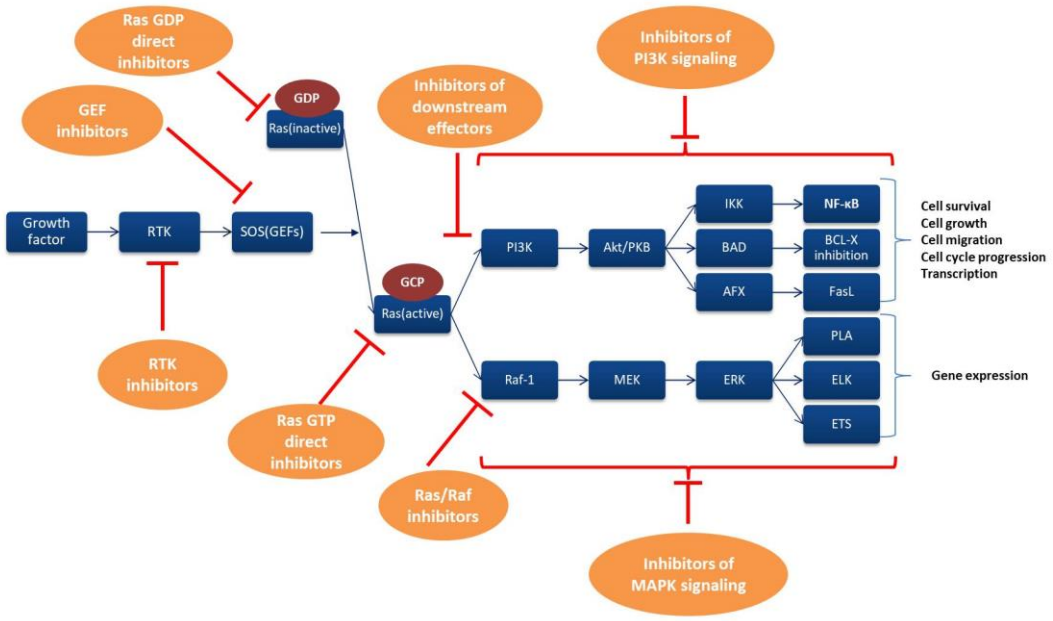

Figure 2. Major Ras signaling pathways. Growth factors can stimulate the Ras signaling pathway. PI3K and Raf pathways are two main Ras-activated pathways that result in vital cell functions. Important strategies to inhibit these signaling pathways and their targets are also depicted.

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There are significant differences in effector binding and activating potential of each family member of Ras proteins. Furthermore, each isoform of Ras proteins interacts selectively with different effectors. For instance, the binding affinity of H-Ras, N-Ras, and K-Ras to Raf kinases is more than TC21, M-Ras, or Rit proteins. The Ras family are also more potent activators of Raf proteins. Besides, Ras isoforms show different activating intensities to Class I PI3K isoforms. N-Ras, H-Ras, K-Ras, R-Ras, M-Ras, and TC21 activate the p110α and p110γ isoforms. R-Ras and TC21 can also activate the p110δ isoform. On the other hand, Ras proteins interact with RalGEFs in similar potency.[17]

2.2.4 K-Ras functional regions

Focusing on the K-Ras isoform, one can suggest the K-Ras structure is constructed of two main parts: hypervariable region (HVR) and G domain. C-terminal region anchored to the plasma membrane is called HVR. However, the G domain (residues 1-166) is the functional domain responsible for GTPase activity. G domain contains six beta-strands as the core of the protein, surrounded by five alpha-helices. There are two regions in the K-Ras structure termed Switch-I and Switch-II bind to the effectors and regulators. These regions are not definite. Switch-I is residues 30 to 40, and Switch II is approximately residues 58–60 to residues 67–76. P-loop region is in contact with the nucleotide called (also known as Walker A motif). P-loop is about residues 10 to 14-17.[30] The detailed structure of K-Ras4B protein is shown in Figure 3. As these region names are arbitrary, residues 10–17, residues 32–38, and residues 59–67 are considered P-loop, Switch I, and Switch II, respectively.

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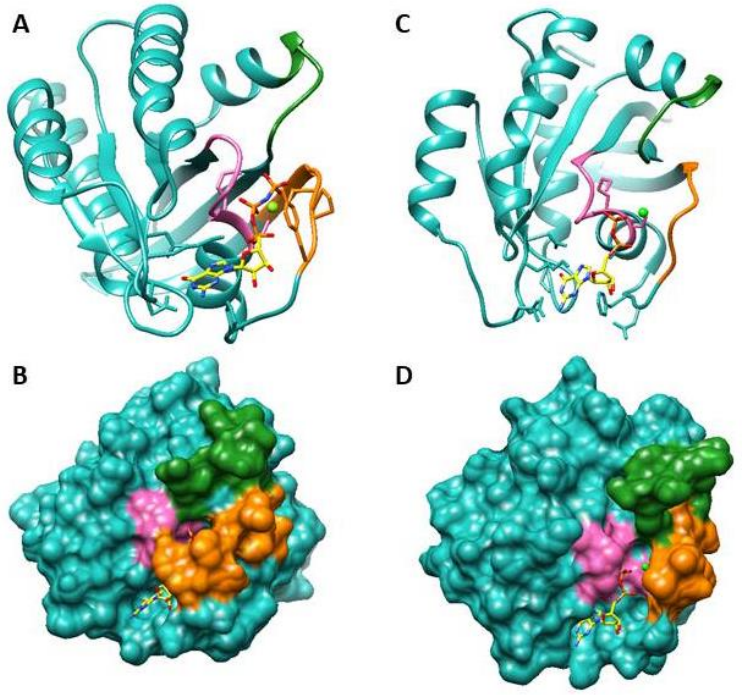

Figure 3. Structure of K-Ras4B. The C-terminal HVR is not present in the structure A) Crystal structure of K-Ras (WT)-GTP. B) Structure of K-Ras (WT)-GTP in surface representation. C) structure of K-Ras (WT)-GDP Selected D) Structure of K-Ras (WT)-GDP in surface representation. Structural regions are highlighted with the following color scheme: P-loop , pink; switch-I , orange; switch-II , green.

The interesting point regarding Ras-effector binding is the β-sheet interaction which can be evaluated for ras activity measurements. Several HRas-GppNHp (the G domain) structures are bound to effectors such as C-Raf, PI3Kγ, RalGDS, NORE1A, PLCε, Grb14, and Bry2 available. Whereas no K-Ras-effector structures of K-Ras or N-Ras have been reported. These structures showed the interaction of the β2-strand of the Ras with an effector ubiquitin-like fold, called RBD (Ras binding domain). Along with the β2-strand, for PI3Kγ, there are interactions with the Switch-II region of Ras. As the G domain of H-Ras and K-Ras are identical, the K-Ras-effector interaction may be similar, as described.[24]

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2.2.5 Ras mutations

The first Ras gene mutated human cancer was identified from the T24/EJ bladder carcinoma cells in 1982.[31] About 27% of human cancers are caused by Ras mutations.[32] The prevalence of K-Ras mutations is the most among the Ras-driven human cancers; however, H-Ras is rarely mutated.[33] Ras mutations dysregulate the signaling of cell proliferation, cell mitosis, and cell apoptosis due to the overexpression of the MAPK and PI3K pathways.[17]

The frequency of each Ras isoform and missense mutations are different in Ras-mutated cancers. K-Ras mutations are the most frequent cause of cancer among other isoforms. The characteristics and function of each isoform define the consequences of its mutations. Stem-like properties of K-Ras-expressing cells (e.g., binding calmodulin), the essential function of K-Ras relative to N-Ras and H-Ras in cellular pathways, rare codons limiting the K-Ras gene expression, and different rate of K-Ras gene repair may be the reasons for increased risk of K-Ras oncogenesis.

Despite inducing several abnormalities, activating mutations of H-Ras in the germline are not fatal. Whereas these mutations are the same as somatic H-Ras activating mutations, they do not cause cancer development. Additionally, total activating alleles of H-Ras can be tolerated in contrast to K-Ras. Variant alleles of K-Ras are rarely seen because of their high oncogenicity. Moreover, the mouse fetus lacking K-Ras dies, unlike the mouse lacking HRas or N-Ras. Therefore, K-Ras has a different role in cell development. Furthermore, the H-Ras knockin fetus of mouse lives, although it develops tumors in response to regular exposure to oncogenes.

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In conclusion, the gene locus of these proteins and differential regulation of H-Ras and K-Ras expression define their importance in cancer development. Additionally, each mutation in individual Ras isoforms leads to specific cellular pathway activation.[34]

Most Ras mutated cancers are due to codons 12, 13, or 61 mutations. Computational analyses have shown that mutations of G12 or G13 to any other amino acids except proline cause a steric hindrance for GAP's arginine finger in the GTPase domain and disrupts the GAP-mediated GTP hydrolysis. Consequently, the concentration of active GTP-Ras form increases in the cell. Moreover, some mutations (e.g., A146) decrease the affinity of nucleotide to Ras. Dissociation of GDP allows the abundant GTP to bind Ras protein.[24]

3. K-Ras targeting treatments

There are two major problems regarding Ras targeting. First, the Inhibition of wild-type Ras could be harmful to normal cells. As a result, approaches that target specifically the Ras mutant are preferred. Second, specific Inhibition of Ras isoforms is crucial since the substitution of inhibited K-Ras with N-Ras or H-Ras is not possible in adults due to the tissue-specific expression of Ras.[35] Consequently, Because of K-Ras allele-specific related cancer types and the vital role of wild-type K-Ras in the cellular function, it is of utmost importance to develop K-Ras mutant specific inhibitors.

Nevertheless, differences in biochemical characteristics of K-Ras mutants will be crucial in determining which Ras state (GDP or GTP) is the aptest for mutant-specific inhibitors.[36] Interestingly, the rate of intrinsic activity varies among different Ras mutants, and the ratio of GDP/GTP states varies as well. Therefore, the specific targeting of each mutant Ras is of utmost importance.[36]

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A Ras protein undergoes a cycle that goes from GDP bound to GTP bound forms. As described before, GEFs accelerate the GDP exchange for GTP. On the contrary, GAP agents mediate GTP hydrolysis. On the other hand, K-Ras's intrinsic GTPase and GDP to GTP conversion activity play a critical part in this cycle. Additionally, the active conformation of K-Ras –due to GTP binding- provides the possibility of effector interacting. Therefore, the downstream effector receives the activating signal.

K-Ras inhibition strategies are categorized as direct and indirect K-Ras targeting. To suppress the K-Ras proteins indirectly, one of the essential steps of K-Ras activation should alter, including the processing and modifications of K-Ras protein, membrane localization, or upstream effector binding (e.g., K-Ras mimicking). However, direct K-Ras inhibition is a more favored approach to treat K-Ras-derived cancers. Blocking any activating steps that include disturbing the active conformation and K-Ras-effector interaction terminates the activating signal. The direct Inhibition of K-Ras can be categorized into 1) the inhibition of nucleotide exchange cycle 2) the direct inhibition of K-Ras–effector interaction. Some important drug candidates designed by described strategies are listed in Table 1.

3.1 The Inhibition of the nucleotide exchange cycle

In 2013 Ostrem et al. designed small molecules irreversibly inhibiting K-Ras(G12C) mutant based on the covalent binding with cysteine 12. Therefore, they did not bind the wild-type K-Ras and exhibited selectivity to G12C mutant. It is shown that there is a groove made by the Switch II and the α3-helix of K-Ras(G12C)-GDP which is not detectable in regular structures of K-Ras (called S-IIP) (Figure 4). The hydrophobic region of these inhibitory molecules binds allosterically to the S-IIP binding pocket. The covalent binding of these inhibitors to Cys 12 in the GDP state of K-Ras blocks the nucleotide exchange and alters the Ras affinity to the nucleotide. Therefore, the preference of GDP over GTP inactivates the K-

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Ras. Furthermore, the SOS meditated nucleotide exchange, and the effector binding is blocked by these compounds. Fragment 6H05 is the original inhibitory molecule that covalently binds K-Ras(G12C)-GDP.[37] ARS-853 is another compound of this group of K-Ras(G12C) inhibitors. This small inhibitory molecule is a modified version of the compound -12 (designed by Ostrem et al.). The acrylamide carboxyl oxygen of the ARS-853 interacts with the Cys 12.[38] Due to the limitations of ARS-853 series modification, some scaffolds were designed that possessed an acrylamide head and a hydrophobic binding site an appropriate distance to interact with S-IIP. The result was quinazoline lead scaffold. The most significant K-Ras inhibitor of this series named ARS-1620. ARS-1620 showed promising selective Inhibition of K-Ras (G12C) mutant tumors *in vivo*.[39]

Moreover, Mirati Therapeutics, Inc. has introduced MRTX849, another cysteine-reactive molecule, with cellular potency of ~10 nM, and its effectiveness on many cancer types with higher selectivity for K-K-Ras(G12C). Therefore, The Mirati company initiated and progressed the Phase 1/2 clinical trials.[40] On the other hand, Amgen company has developed a similar drug candidate to ARS-1620, named AMG 510, targeting the K-K-Ras(G12C) mutant and also initiated a Phase 2 clinical trial.[41] The described compounds are promising candidates for direct and selective Inhibition of K-Ras (G12C) mutant, and whichever achieves the regulatory approval will be the first anti-K-Ras medicine in the clinic.

As described previously, the K-Ras (G12C) is only a portion of K-Ras mutations. The positive point in this mutation is the presence of cysteine, which makes the disulfide bond possible between the compounds and K-Ras protein. However, other approaches are necessary for effective Inhibition of other K-Ras mutations, particularly G12D and G12V. For example, in one of the efforts, a peptide, termed KRpep2d, was designed by phage display and was shown to inhibit K-Ras (G12D) selectively.[42] However, not complete

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specificity toward mutant K-Ras was the main drawback and reason that it could not enter a trial. However, our group studied computationally on its sequence optimization to increase specificity (data not published). In another study of our group, the mechanism of this peptide was thoroughly investigated by molecular dynamics simulation, and it was revealed that following KRpep2d binding to K-Ras (G12D), the GTP molecule dislocates from P-Loop. This observation was not the case in other variants of K-Ras protein (data not published).

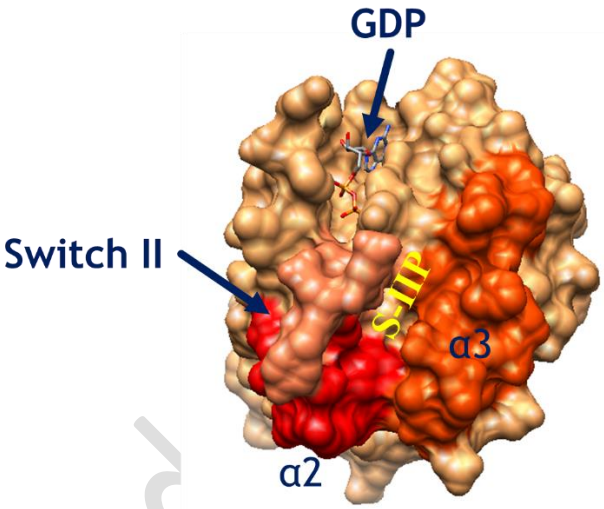

Figure 4. The S-IIP of the K-Ras (G12D)-GDP made by α2, α3, and Switch II (PDB: 5XCO).

Another approach to inhibit the nucleotide exchange is to develop synthetic peptide antagonists with micromolar binding affinity, based on an α-helix from SOS. In this approach, the αH helix, the SOS helix that contacts directly with K-Ras, is mimicked to occupy the K-Ras-SOS interaction site and avoid K-Ras-SOS's direct contact [43,44] (Figure 5).

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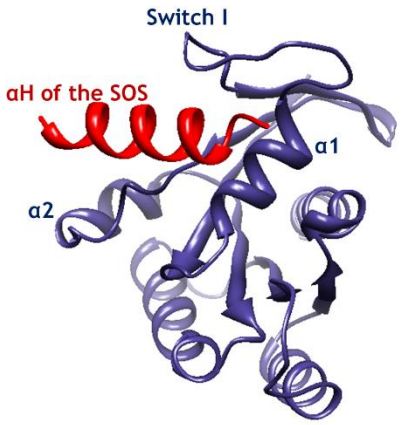

Figure 5. The complex of H-Ras-GTP and the αH domain of SOS (PDB:1NVV). H-Ras and SOS are shown in blue and red, respectively.

HBS3 is one of these peptides that bind the nucleotide-free Ras selectively and reduce nucleotide exchange and Ras signaling.[43] On the other hand, Walensky developed SAH-SOS1 stapled peptide and showed in vitro binding to Ras with unclear specificity to Ras isoforms.[44] Since the mentioned peptides could not move forward to clinical trials, the studies were shifted to small molecule Ras-SOS inhibitors. One of the partially successful compounds was BAY-293 which showed a growth inhibition effect when combined with ARS-853 in a K-Ras(G12C) mutant cell line.[45] This result suggests that these types of compounds can be used with direct K-Ras-GDP (g12C) inhibitors.

3.2 The inhibition of Ras–effector interaction

Some other research groups focused on the Inhibition of Ras-effector interaction. One of the first efforts was the examination of sulindac, as an NSAID, and its derivatives. They were able to block the Raf activation and consequently decreased the tumor transformation.[46] Despite the positive result, their potency was insufficient.[47] Other unsuccessful efforts were developing a cell-penetrating peptide termed cyclorasin 9A5 and a compound named MPC1. Both lacked potency and selectivity to Ras isoforms.[48,49] The cyclic Cyclorasin9A5 peptide

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binds the GTP form of Ras and decreases the cell growth by blocking the Ras-effector interaction.[49] More screenings led to other small molecules designed against Ras-GTP called Kobe0065 and Kobe2601. They were designed based on the Raf structure to inhibit the Ras-Raf interaction. These molecules bound near the effector binding domain and showed inhibitory effect on HRas(G12V)-GTP signaling to downstream effectors.[50]

In an effort to identify additional RAS-RAF interaction inhibitors, a yeast two-hybrid screen has been carried out.[48] RAS protein is attenuated by these compounds in NIH3T3 cells harboring Ras (G12V) mutant.[51] In addition, the compounds reverse several RAS-mutant cell lines' transformative phenotypes and suppress ERK phosphorylation. There is still uncertainty over whether these molecules directly bind to RAS or RAF, as with sulindac compound, and because structural data has yet to be generated, further chemical optimization is challenging.

Most recently, Zhang and colleagues developed cyclic peptides based on the Ras-Raf binding site.[52] The peptides show selective Inhibition of K-Ras (G12D) in GTP form over the wild-type K-Ras. This selectivity is a significant advantage since the K-Ras (G12D) is mostly in GTP state and the wild-type K-Ras is necessary for normal cell proliferation. The authors also investigated the crystal structure of the peptides bound to K-Ras (G12D). It was shown that the peptide KD2 binds in the Switch II groove and α2 helix in a way that residue 12 has direct contact with aspartic acid 12 of K-Ras (G12D). This study's result was promising to focus on the structural and dynamicity of K-Ras protein for drug discovery in this field.

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3.3 Drawbacks of Ras inhibition

The main pitfall of Ras direct inhibition to treat Ras-driven cancer types is that some possible resistance mechanisms might take place. For example, some intrinsic mechanisms of resistance may happen due to the heterogeneity of tumors. For instance, if a tumor contains both a high percentage of K-Ras (G12C) and a lower percentage of K-Ras (G12V) cells, after the treatment with a K-Ras (G12C) inhibitor, gradually the K-Ras (G12V) cells will be selected, and the tumor could relapse. Besides, any intrinsic resistance to K-Ras (G12C) inhibitor can occur in heterogenic tumor cells.[53] However, there is slight evidence that which kind of mutations can arise after the treatment with specific K-Ras (G12C) inhibitors.

Another possible mechanism of resistance is the GTPase activity disabling mutations of Ras proteins. A confirming study showed that resistance occurs following the loss of NF1 protein.[54] Furthermore, a GTPase inactivating mutations such as A59G occurs beside to G12C mutation, K-Ras (G12C) inhibitors effect reduces since K-Ras (G12C) is mainly in GTP state.[55] Nevertheless, the consistency of such phenomenon is unclear across *RAS* mutations in various cancer types.

On the other hand, the direct targeting of GTP-bound Ras can lead to drastic changes of Ras-GTP known conformation and effector binding. As a result, presumably, the possible resistance mechanism to K-Ras (G12C) inhibitors in GDP-bound K-Ras proteins is reduced.

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Table 1. Direct therapeutic strategies for K-Ras mutant cancers.

Category	Target/mechanism	Compound	Origin	Ref
Direct allosteric Ras inhibitors	K-Ras(G12C)/ irreversible covalent inhibitors	Sotorasib (AMG510)	Modification of ARS 1620	41
		MRTX849	Synthetic libraries	40
		ARS-1620	Modification of ARS 853	56
	K-Ras(G12D)	KRpep2d	Phage display	42
K-Ras-effector interaction inhibition	Ras-mimetic	Rigosertib		57
	K-Ras-G12V-effector interactions	Cyclorasin 9A5	Synthetic cyclic library	58
		Peptide 49	Synthetic bicyclic library	59
	K-Ras-G12D-Raf interactions	KD2	*in vitro* translation−mRNA display technology	52
	Post translational modification of Raf (Decreased affinity of C-Raf to Ra)	PRMT6	Arg 100 methylation in C-Raf	60
	Raf dimers	Peptide 38	BRaf residues 505-518	61
		Tat-braftide	BRaf residues 508-517	62
Nucleotide exchange cycle inhibition	SOS helix mimic	HBS3	SOS1 residues 929-944	59
		SAH-SOS1A	SOS1 residues 929-944	44

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4. Conclusion and Future perspective

The most important purpose in Inhibition of the aberrant Ras signaling is blocking the binding of mutated Ras and downstream effectors like Raf and PI3K. The final goal of the prevention Ras from switching to the active conformation is to disrupt the downstream effector binding and, therefore, muting the signaling pathway. Thus, blocking the GTP-bound state of the Ras mutant can be an inhibition strategy only in the case of resulting in the formation of the inactive conformer.

So far, the most successful Ras targeting drug candidates were developed for K-Ras (G12C), which increases hope for the treatment of Ras-driven cancer types. However, there is still a long way to develop specific inhibitors of other Ras mutant variants such as K-Ras (G12V) and K-Ras (G12D) since these alleles are the most frequent K-Ras mutations with the larger associated group of cancer patients. Although the Ras mutant specific therapies seem ideal for personalized medicine and fewer side effects, they possibly have lower efficacy as single-drug therapy. Therefore, the combination therapy may increase Ras mutant-specific inhibitors' efficiency. Because Ras (G12C) specific drug candidates are close to getting approval and also there is a high number of peptidic and small molecule compounds in the clinical pipelines, we can hope to treat Ras mutant cancers finally.

The Inhibition of Ras nucleotide exchange was unsuccessful *in vivo*, with one exception of BAY-293 in a combination of ARS-853. Also, a SOS1 specific inhibitor, as a single and combination therapy, will progress in a clinical trial/phase I [36]. Moreover, the suppressing of the Ras downstream pathway did not show clinical benefit. However, many combinational treatments are going on in clinical trials.

Ras was thought to be undruggable due to a lack of information about the S-IIP pocket of the Ras-GDP. The Discovery of the S-IIP heartened the researchers to find new anti-Ras

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candidates. At the same time, unaided targeting the S-IIP may not build sufficient affinity to Ras. More significant compounds having suitable hydrophobic and hydrophilic domains can increase the ability of Ras-binding.

In conclusion, we are not sure if the GDP-bound form of the K-Ras mutants is inactive. Inactivation of the Ras mutants depends on their capability of effector binding. Therefore, it seems that selective Ras mutant-effector interaction blocking is a promising approach for terminating the aberrant signal of growth. Moreover, there is no certainty about the mechanism of action of the Ras blocking candidates. The binding of the anti-Ras small molecules and peptides alters the conformation of the Ras, including the switches and the effector binding site leading to the possibility of interaction with the downstream effector. In summary, targeting the effector critical regions of Ras or changing the active conformation of Ras to impact the effector binding site may be the logic of the future design of anti-Ras candidates.

Conflict of Interests

The authors claim that there is no conflict of interest.

Author's Contributions

JPA, AB, MSH and EMA designed the idea of the manuscript. JPA, AB, MSH, NH, HRH and EMA wrote and revised the manuscript.

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