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Perspective

# Inhibition of Nonfunctional Ras

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## SUMMARY

Intuitively, functional states should be targeted; *not* nonfunctional ones. So *why could drugging the inactive K-Ras4B$^{G12C}$ work—but drugging the inactive kinase will likely not*? The reason is the distinct oncogenic mechanisms. Kinase driver mutations work by stabilizing the active state and/or destabilizing the inactive state. Either way, oncogenic kinases are mostly in the active state. Ras driver mutations work by quelling its deactivation mechanisms, GTP hydrolysis, and nucleotide exchange. Covalent inhibitors that bind to the inactive GDP-bound K-Ras4B$^{G12C}$ conformation can thus work. By contrast, in kinases, allosteric inhibitors work by altering the active-site conformation to favor orthosteric drugs. From the translational standpoint this distinction is vital: it expedites effective pharmaceutical development and extends the drug classification based on the mechanism of action. Collectively, here we postulate that drug action relates to blocking the mechanism of activation, not to whether the protein is in the active or inactive state.

## BACKGROUND

Why can targeting inactive Ras conformations inhibit active ones, but targeting inactive kinase conformations likely will not? There are already examples of inhibition of inactive (nonfunctional) mutant K-Ras4B$^{G12C}$ (Goody et al., 2019; Janes et al., 2018; Kettle et al., 2020; Lou et al., 2019; McCarthy et al., 2019; Nagasaka et al., 2020; Nussinov and Tsai, 2015; Ostrem et al., 2013; Zeng et al., 2017), among them some with promising drugs undergoing clinical trials, and ongoing attempts to tame K-Ras dimers (Kessler et al., 2020). However, inhibition of nonfunctional kinases was not taken up. This is vastly important to understand, as allosteric inhibitors are increasingly becoming a major drug-discovery route (Gross et al., 2020; Khan et al., 2020b; Kostaras et al., 2020; Lu et al., 2018, 2020; Nussinov and Tsai, 2012, 2013, 2015; Ward et al., 2020). Here, we first briefly review Ras biology, highlighting key attributes in Ras activation, some of which have been pursued in Ras pharmacology attempts. We follow with allosteric mutations and inhibitors aimed at nonfunctional Ras states, successes, failures, and considerations. We distinguish these from oncogenic activation of kinases and consequent allosteric inhibitor strategies. In Ras, activating mutations work by interfering with deactivation; in kinases, they work by increasing the relative stability of the active versus the inactive state, thus vastly increasing the number of molecules in the active state. It is exceedingly challenging to design a high-affinity drug that would bind the inactive state and shift the ensemble back to this state while still at nontoxic dosage. Allosteric drugs that alter the active site appear more tractable.

However, the location of the drug-binding site matters (Guarnera and Berezovsky, 2019, 2020), and in kinases the inactive state may also be targeted. For example, Raf's activation is through homo- or heterodimerization. If a drug binds at the dimer interface to prevent activation via dimerization, it is a successful inhibitor of a nonfunctional kinase conformation. On the other hand, an inhibitor of active guanosine triphosphate (GTP)-bound Ras, binding at the interface of the effector binding site, still prevents the signal transduction of active Ras. That is, *the drug action is related to blocking the protein mechanism of activation*, not to whether the protein is in the active or inactive state. That is, we suggest drug classification based on its mechanism of action *as it would help guide its design*.

## RAS: AN OVERVIEW

Wild-type Ras regulates cell growth and division (Bryant et al., 2014; Cox and Der, 2010; Crespo and Leon, 2000; Drosten et al., 2010; Lu et al., 2016b; Pylayeva-Gupta et al., 2011; Wiesmuller and Wittinghofer, 1994). An activated receptor tyrosine kinase (RTK), such as epidermal growth factor receptor (EGFR) (Moran et al., 1991; Nussinov et al., 2020), recruits the Son of Sevenless (SOS), a Ras-specific guanine nucleotide exchange factor (GEF). Mediated by adaptor proteins SHC (Src homology 2 domain containing) and Grb2 (growth factor receptor-binding protein 2), RTK activates SOS, which acts to substitute guanosine diphosphate (GDP) by GTP at the membrane to activate Ras (Bandaru et al., 2019; Huang et al., 2019; Liao et al., 2018, 2020). Membrane-anchored Ras dimers or nanoclusters bind


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and activate Raf kinase (Nussinov et al., 2019b, 2019c, 2020; Solman et al., 2015; Sutton et al., 2019; Zhou et al., 2018). In the absence of Ras, Raf is autoinhibited. Its Ras-binding domain (RBD) and cysteine-rich domain (CRD) loosely interact and occlude the kinase domain (KD) dimerization surface (Nussinov et al., 2018b). This conformation is stabilized by 14-3-3 (Kondo et al., 2019; Park et al., 2019; Rezaei Adariani et al., 2018). The high-affinity Ras-Raf's RBD interaction, enhanced by CRD membrane attachment (Fang et al., 2020; Li et al., 2018a, 2018b; Okada et al., 1999; Sarkar and Garcia, 2020; Travers et al., 2018), shifts Raf's equilibrium toward the open state and interaction with Ras (Nussinov et al., 2019a). RBDs' interactions with spatially proximal Ras catalytic domains in Ras dimers or nanoclusters promote Raf's KD dimerization and activation (Holderfield et al., 2014; Lavoie et al., 2013; Lee et al., 2019; Muratcioglu et al., 2015, 2020). Active Raf phosphorylates MEK1/2 (MAPK kinase 1/2) (De Luca et al., 2012; Young et al., 2013), with signaling propagating down the mitogen-activated protein kinase (MAPK) pathway to activate extracellular signal-regulated kinase (ERK) and transcription factors, such as Elk-1 (Gao et al., 2019; Nussinov et al., 2014), with the resulting expressed proteins entering the $G_1$ phase of the cell cycle, thereby linking the cellular environment to cell-cycle progression. At the $G_1$ phase, cyclin-dependent kinases promote passage to the S (synthesis) phase.

The second major pathway activated by Ras at the membrane is Ras/PI3K/AKT/mTOR (Downward, 2008; Sheridan and Downward, 2013). RTK interacts with the nSH2 domain in the p85$\alpha$ regulatory domain of phosphatidylinositol 3-kinase (PI3K$\alpha$), recruiting the lipid kinase to the membrane and releasing its autoinhibition (Zhang et al., 2019a, 2019b). Subsequent conformational changes expose the active site (Zhang et al., 2019a). Ras-GTP assists in the recruitment, acting to enhance the catalytically favored PI3K population at the membrane where signaling lipid substrate phosphatidylinositol-3,4-bisphosphate ($PIP_2$) is located. PI3K phosphorylates $PIP_2$ to produce phosphatidylinositol-3,4,5-bisphosphate ($PIP_3$). $PIP_3$ recruits AKT (also known as protein kinase B [PKB]) and 3-phosphoinositide-dependent protein kinase 1 (PDK1) to the plasma membrane. PDK1 phosphorylates and activates AKT, with the signaling propagating downstream to activate transcription factors whose transcription products also enter the cell cycle at the $G_1$ phase. Together with the MAPK products, they act in cell growth and division, accelerating proliferation (Gul et al., 2018; Lien et al., 2017; Nussinov et al., 2019a, 2020; Pokrass et al., 2020; Zhang et al., 2020). Countering $PIP_2$ to $PIP_3$ phosphorylation by PI3K is PTEN (phosphatase and tensin homolog), a phosphatase (Hosford et al., 2017; Ijuin, 2019; Yehia et al., 2020).

Ras anchors in the plasma membrane through its prenylated hypervariable region (HVR) at the C terminus (Barklis et al., 2019; Nussinov et al., 2018a). Whereas the sequences and structures of the catalytic domains of H-Ras and N-Ras, and splice isoforms K-Ras4A and K-Ras4B, are highly similar, this is not the case for their HVRs (Lu et al., 2016b; Nussinov et al., 2017a; Pylayeva-Gupta et al., 2011) and the distinct combinations of their lipid post-translational modifications—farnesyl (or geranylgeranyl) and palmitoyl. K-Ras4B is farnesylated, K-Ras4A can be farnesylated or farnesylated and palmitoylated; N-Ras is also farnesylated and palmitoylated, and H-Ras is farnesylated and double-palmitoylated. Their distinct chemistry makes them favor different membrane properties and, thus, segregation into homogeneous nanoclusters and dimers (Fujiwara et al., 2002; Murase et al., 2004; Simons and Vaz, 2004). It also influences Ras orientation at the membrane, accessibility, and selective interaction with its effectors (Ahearn et al., 2011; Cao et al., 2019; Chavan et al., 2015; Jang et al., 2016a, 2020; Neale and Garcia, 2020; Nussinov et al., 2018a; Prakash and Gorfe, 2019; Prakash et al., 2020). Membrane attachment can also be regulated by ubiquitination (Baietti et al., 2016; Steklov et al., 2018; Thurman et al., 2020), phosphorylation of the C terminus as well as other post-translational modifications, such as acetylation, and calmodulin (Jang et al., 2017, 2019; Nussinov et al., 2017b).

As members of the small GTPase family, Ras proteins function as molecular switches between their two states (Bourne et al., 1991; Cherfils and Zeghouf, 2013; Downward, 1990; Geyer and Wittinghofer, 1997; Grand and Owen, 1991; Lamontanara et al., 2014; Lowy et al., 1991; Sprang, 1997; Takai et al., 2001; Vetter and Wittinghofer, 2001; Wittinghofer and Pai, 1991; Wittinghofer and Vetter, 2011). Inactivation is mediated by GTPase-activating proteins (GAPs) that hydrolyze the GTP to GDP (Wittinghofer et al., 1997). Strong oncogenic mutations block GAP catalytic activity, retaining the active GTP-bound state, thus promoting downstream signaling and cell proliferation as in the case of G12D K-Ras4B mutant (Adjei, 2001; Schubbert et al., 2007; Smith and Ikura, 2014; Smith et al., 2013; Vigil et al., 2010). Weaker driver mutations can be associated with promoting a high rate of intrinsic and guanine nucleotide exchange factor-induced nucleotide exchange, as in the case of A146T K-Ras4B mutant (Poulin et al., 2019).

Below, we first distinguish between allosteric mutations in kinases and in Ras. In Ras, they work by quenching deactivation mechanisms, GTP hydrolysis, and nucleotide exchange; inhibitors that bind to the inactive conformation block reactivation. By contrast, in kinases they act by shifting the equilibrium toward the active state; accordingly, their allosteric drugs aim to modulate the active-site conformation. The distinct mutation scenarios are the key to their inhibition strategy, and these considerations are general.

## ALLOSTERIC MUTATIONS IN RAS AND KINASES WORK DIFFERENTLY; THUS, THEIR INHIBITION STRATEGIES SHOULD DIFFER TOO

Protein function hinges on the extent to which it populates its active conformation (Lu et al., 2014a, 2014b; Nussinov and Tsai, 2013). Molecular ensembles exist in a conformational equilibrium of active and inactive states (Henley et al., 2020; Nussinov, 2016; Nussinov and Wolynes, 2014). Aside from repressors, under physiological conditions most enzymes and receptors are in their inactive states. When stimulated, either by an incoming signal or oncogenic mutations, the ensemble shifts to populate the active state (Tsai and Nussinov, 2014). The relative populations of the states reflect their relative stabilities. Allosteric inhibitors whose shapes and chemistry fit a certain patch of surface of the enzyme or receptor in the inactive (or for repressors, active) state are conformationally selected. The ensemble is equilibrated by a shift toward the stabilized inactive state.

Kinases fit into this description (Figure 1). They switch from the inactive $\alpha$C-helix-out to the active $\alpha$C-helix-in conformation

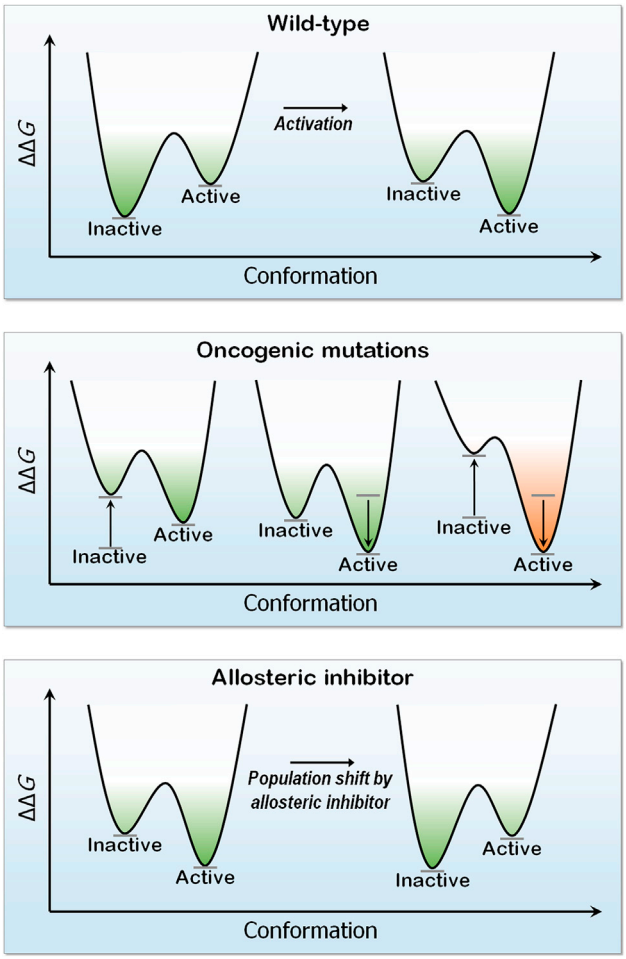



**Figure 1. The Free Energy Landscape ΔΔ*G* of a Kinase Conformation (here EGFR), the Effect of Mutations, and an Allosteric Inhibitor**

Oncogenic driver mutations (L858R and T790M) destabilize the inactive state (left plot in the middle panel) and stabilize the active state (center plot), or both (right plot). The outcome is a shift of the ensemble toward the constitutively active EGFR conformation. In the bottom panel, an allosteric drug binding EGFR's mutant surface stabilizes this conformation, with a population shift toward this conformation. The drug modulates the active-site shape.

through a rotation and shift movement, with a salt bridge between the β3-Lys and the αC-Glu, and R-spine formation (Nussinov and Tsai, 2013). The triggers for the conformational change vary among the kinase families. Oncogenic mutations stabilize the active αC-helix-in conformation or disrupt interactions that stabilize the αC-helix-out, as in the case of the L858R in EGFR. There, the mutation stabilizes the αC-helix-in conformation by heterodimerization. The mutation is in the middle of a hydrophobic core. Thus, the change to Arg also disrupts stabilizing hydrophobic interactions in the αC-helix-out conformation. The T790M mutation in EGFR, T315I in Bcr-Abl, T334I in c-Abl, T341I in Src, T670I in Kit, and T674I in PDGFRα (platelet-derived growth factor receptor A) work by stabilizing the hydrophobic R-spine, promoting enzymatically active conformation (Nussinov and Tsai, 2013). In another example, in PI3Kα lipid kinase (Zhang et al., 2019a) oncogenic mutations E542K and E545K in the helical domain, with charge reversal (Leontiadou et al., 2018), mimic the release of the autoinhibition. In the wild type it is triggered by nSH2 domain interacting with RTK's pYXXM motif in the C terminus. The outcome is exposure of the active site at the membrane. Other mutations, e.g., Glu81, Gly106, Arg108, Lys111, and Gly118, in the adaptor-binding domain, contribute to the exposure as well. These mutations reduce the transition-state barrier ($k_a$). Another common driver, H1047R, enhances the population time of the lipid substrate at the active site ($k_m$). In TRK tyrosine kinases, xDFG mutations confer resistance to type I inhibitors but sensitize them to type II by stabilizing the inactive (DFG-out) conformations (Cocco et al., 2020).

Not all mutations can, however, be described by the free energy landscape. Ras's major mutations, e.g., Gly12, Gly13, and Gln61, which block hydrolysis, cannot be described by the free energy landscape (Fernandez-Medarde and Santos, 2011; Nussinov et al., 2013; Tsai and Nussinov, 2014). Neither can mutations that block nucleotide exchange, such as K-Ras4B$^{A146T}$. GTP hydrolysis involves proton transfer from an attacking water molecule to another, followed by transfer of a different proton from this water molecule to the GTP. Gln61 can stabilize the transient $OH^-$ and $H_3O^+$ molecules lowering the transition-state barrier, which the Leu61 cannot (Martin-Garcia et al., 2012). As to the Gly12 mutants, their longer side chains interfere with GAP's arginine finger insertion and hydrolysis (Krengel et al., 1990).

Despite the different mechanisms, both kinases and Ras GTPases are dynamic molecules, with multiple, not only two discrete, active and inactive states. Both types of proteins can be considered from the mechanistic free energy landscape standpoint, which epitomizes the "second molecular biological revolution" (Nussinov and Wolynes, 2014). The paradigm fomented by this revolution postulated that the energy landscape (Frauenfelder et al., 1991) and the laws of quantum mechanics and structural chemistry apply to all biological systems and that biomolecules always interconvert between conformations with varying energies. This view inspired the dynamical statistical description of the molecules (Kumar et al., 2000; Ma et al., 2002; Tsai et al., 1999a, 1999b), whereby the ensembles describe the relative conformational stabilities of the states, their transitions, and their population shifts to retain their equilibrium (Arora and Brooks, 2007; Boehr et al., 2009; Huang et al., 2015; Kar et al., 2010; Mackereth et al., 2011; Motlagh et al., 2014; Nussinov and Ma, 2012; Okazaki and Takada, 2008). GDP-bound Ras can exist in inactive and active conformations. Similarly, Ras-GTP exists in active and inactive states, although the populations vary. Indeed, molecular dynamics (MD) simulations and crystallography (Shima et al., 2010) suggest that the inactive state of Ras-GTP may exist in at least three substates. Notably, in reality, there are many inactive protein states and there is also an ensemble at the transition state. Classically, catalysis involves tight binding of the substrate to the transition-state structure to lower the activation energy. However, the transition state is a surface, not a single saddle point on the potential energy surface, and the activated conformations in the transition-state ensemble can catalyze multiple reaction steps (Ma et al., 2000). Nuclear magnetic resonance of GppNHp-bound wild-type H-Ras catalytic domain identified two conformational states that interconvert on a millisecond timescale, inactive state 1 and active state 2 (Geyer et al., 1996; Liao et al., 2008). The perturbations induced by the oncogenic mutations promote a redistribution of the

ensemble. Crystal structures reveal allosteric effects on conformational states induced by mutations, and mutants display distinct outcomes in effector binding and in oncogenicity, as in the cases of G12V versus G12D. Furthermore, K-Ras populates conformational states differently from its isoform H-Ras and oncogenic mutant K-Ras4B$^{G12D}$ (Parker and Mattos, 2018; Parker et al., 2018).

The free energy landscape describes the ensemble around the native state as populating the active and inactive states, with the states separated by kinetic barriers. The more stable the conformation, the larger its population. Due to functional requirements, the barriers between states are not too high, which allows conformations to switch between them. In kinases, oncogenic mutations work by creating new interactions and/or abolishing existing ones, resulting in higher stability (population) of the active state. In the case of Ras, these free energy considerations do not apply to the mutation actions. GTP binds the active state. With the high-millimolar-range concentration of the guanine nucleotide in the cell and its picomolar affinity, ~75% of K-Ras$^{G12C}$ is GTP bound in the steady state. Intrinsic hydrolysis, which is high for this mutant (Hunter et al., 2015), cycles K-Ras4B$^{G12C}$ into its GDP-bound state while allosteric drugs block the nucleotide exchange.

Thus, not all mutations can be described by the free energy landscape; only those working via a population shift can (Tsai and Nussinov, 2014). Accordingly, the nature of the drugs should be different. The nonfunctional states of Ras can be targeted; however, for kinases, shifting the ensemble away from the constitutively active state, by for example stabilizing the inactive autoinhibited state, is challenging.

Imatinib (STI-571/Gleevec), the first synthetic kinase inhibitor to target an oncogene (Bcr-Abl), can serve as a classical example to illustrate our point (Nagar et al., 2003). The physiological state of Abl is autoinhibited via intradomain interactions between the SH3-linker-SH2 and the KD. The fused Bcr abolishes the autoinhibition to constitutively activate it. The landmark crystal structure of the KD described by Kuriyan and colleagues with imatinib bound at the ATP-binding site reveals a collapsed activation loop blocking substrate binding, which is open and extended in all *active* KDs. This binding indicates that imatinib only can bind to the less populated, *inactive* kinase conformation of Bcr-Abl. The drug-resistant mutations observed by Daley and colleagues (Azam et al., 2003) either directly participate in imatinib binding or act allosterically to destabilize the autoinhibited Abl kinase conformation to which STI-571 preferentially binds. That is, they reduce drug-binding affinity or increase the population of the active state by destabilizing the inactive conformation, counteracting the inhibiting action of imatinib.

However, as the inhibition of Raf (homo- or hetero-) dimerization shows, the location of the drug binding matters: if the drug is bound at the dimer interface to prevent activation via dimerization, it is a successful inhibitor bound to an inactive kinase conformation.

## APPROACHES TO RAS INHIBITION

Driver mutations in K-Ras are common in cancers (Chen et al., 2015; Eser et al., 2014; Holderfield et al., 2014; Krens et al., 2010; Prior et al., 2012; Stephen et al., 2014); however, the massive community efforts to identify effective inhibitors have proved to be challenging (Cox et al., 2014; Ledford, 2015; Lu et al., 2016c; Milroy and Ottmann, 2014; Samatar and Poulikakos, 2014; Shao et al., 2014; Spiegel et al., 2014; Stites and Ravichandran, 2009; Wang et al., 2013; Zimmermann et al., 2013). There are many reasons for the difficulty, including Ras's picomolar affinity for guanine nucleotide whose cellular concentration is in the millimolar range, making binding of nucleotide analogs disfavored (Khan et al., 2020a; Waters and Der, 2018). In addition, despite searches, deep pockets for small-molecule binding have not been identified (Spiegel et al., 2014). The diverse strategies of potential Ras inhibitors have been reviewed extensively in the literature (Chatani and Yang, 2020; Cox et al., 2014, 2015; Dang et al., 2017; Gentile et al., 2017; Gorfe and Cho, 2019; Gupta et al., 2019; Khan et al., 2020a; Liu et al., 2010, 2019; Lu et al., 2016a, 2016c; Mattingly, 2013; McCormick, 2018; Mullard, 2019; O'Bryan, 2019; Papke and Der, 2017; Papke et al., 2016; Patricelli et al., 2016; Sakamoto et al., 2017; Sheridan, 2020; Singh et al., 2015; Spencer-Smith and O'Bryan, 2019; Spiegel et al., 2014; Zhang and Shokat, 2019; Zimmermann et al., 2013). Among those that have been explored are agents blocking Ras dimerization (e.g., NS1 and K13) (Spencer-Smith et al., 2017) and inhibition of farnesyl transferase (FTase), thus blocking cysteine farnesylation at the C terminus, translocation from the ER to the plasma membrane, and anchorage (e.g., tipifarnib, deltasonamide) (Baranyi et al., 2020; Cheng et al., 2020; Martin-Gago et al., 2017; Reid and Beese, 2004). Blocking Ras-GEF interaction (e.g., DCAI, HBS3) (Maurer et al., 2012; Patgiri et al., 2011), and Ras-Raf (e.g., Abd-7, compound 3344) (Bery et al., 2018; Quevedo et al., 2018) (Figure 2) have also been explored. In another strategy, an unbiased approach using synthetic single-domain monobodies (Spencer-Smith et al., 2017) was employed. An NS1 monobody for Ras isoforms selectively inhibited oncogenic H-Ras and K-Ras, but not N-Ras, proliferation (Khan et al., 2020a). Additional strategies for preventing Ras-plasma membrane anchorage include enhancing K-Ras phosphorylation, inhibiting K-Ras interaction with its chaperone protein, and more (Cheng et al., 2020; Gorfe and Cho, 2019). With the aim of identifying functionally productive states with the effector interacting surface exposed, Ras orientation at the membrane has also been considered (Cao et al., 2019; Chavan et al., 2015; Jang et al., 2016a, 2020; Neale and Garcia, 2020; Nussinov et al., 2018a; Prakash and Gorfe, 2019; Prakash et al., 2020). These approaches are hindered, however, by membrane dynamics and fluidity, toxicity, specificity, and thus altogether effectiveness. SML-8-73-1 (Hunter et al., 2014; Lim et al., 2014; Xiong et al., 2017) and SML-10-70-1 (Lim et al., 2014) are covalent small-molecule inhibitors binding at the active site of oncogenic G12C K-Ras mutant. Direct approaches of targeting Ras appear more promising but still face hurdles. Early on, a small-molecule compound Kobe0065 and cyclen-metal complexes that bound to the inactive state were developed (Shima et al., 2013). A covalent allosteric inhibitor for oncogenic K-Ras$^{G12C}$ obtained by a disulfide fragment-based screening approach (Ostrem et al., 2013) as well as inhibitors that target nonfunctional dimers (Kessler et al., 2019, 2020; Tran et al., 2020) exploit the same rationale.

Allosteric compounds that target inactive monomers and dimers may hold the key for future successes. Nonetheless,

targeting dimers may also face additional hurdles: spatially proximal Ras nanoclusters can play a major role in MAPK activation; however, PI3K activation does not require Ras dimers, so its activation can still go through (Nussinov et al., 2018a, 2019b).

## ALLOSTERIC DRUGS TARGETING NONFUNCTIONAL RAS

Using a fragment-based screening approach, Shokat and coworkers pioneered the first compounds that covalently link to the cysteine in GDP-bound K-Ras$^{G12C}$, blocking SOS-catalyzed nucleotide exchange (Ostrem et al., 2013). Their initial leads had only millimolar affinity, although subsequent work by several groups led to other high-affinity drugs with some undergoing clinical trials (Sheridan, 2020). Among the more potent compounds are AMG510 (Sotorasib) by Amgen (phase I trial), MRTX849 (phase I/II) (Canon et al., 2019; Hallin et al., 2020; Mirati Therapeutics, 2020), JNJ-74699157 (formerly ARS-3248) (phase I, Johnson & Johnson, Wellspring Biosciences; earlier ARS-1620 [Janes et al., 2018]), and LY3499446 (phase I/II clinical trials, Eli Lilly). Illustrating the hardships in developing a drug against K-Ras, Eli Lilly exited the K-Ras race due to toxicity in its phase I effort, and Johnson & Johnson stopped recruiting (Adams, 2020). Initial results in patients with K-Ras$^{G12C}$ mutation indicated that MRTX849 is a promising clinical candidate (Fell et al., 2020; Gabizon and London, 2020). Both AMG510 and MRTX seem to be moving at equal pace in the clinic. Phase I data for AMG510 in advanced colorectal cancer suggested that monotherapy provided prolonged disease control. Antitumor activity was also observed across multiple solid tumors (Hong et al., 2020). A phase II monotherapy study in advanced colorectal cancer is getting under way. A targeted degradation strategy for oncogenic K-Ras$^{G12C}$ by construction of a library of C12-directed covalent degrader molecules (PROTACs) was also explored; however, initially the lead degrader was unable to effectively polyubiquitinate cellular K-Ras$^{G12C}$ (Zeng et al., 2020). More recently, LC-2, a PROTAC capable of degrading endogenous K-Ras$^{G12C}$ via E3 ligase VHL, was developed. It too covalently binds K-Ras$^{G12C}$ with an MRTX849 warhead, resulting in MAPK suppression (Bond et al., 2020). These allosteric compounds bind to nonfunctional K-Ras$^{G12C}$.

The covalent attachment first designed by Shokat and colleagues involved disulfide tethering to the Cys, exploiting the Cys thiol reactivity (Ostrem et al., 2013). Exploiting this method, they obtained compounds that bound in an unknown pocket in the Switch II region near the nucleotide binding pocket (SW2 binding pocket). The pocket exists in the GDP-bound but not in the GTP-bound conformation; thus, these compounds bound only the GDP-bound state. Their binding resulted in conformational changes in the Switch I and Switch II regions, inhibiting interactions with Ras effectors and activators. The impact of this work resulted from its significant and novel components: it was the first to exploit covalent allosteric drug on Ras and it exploited its inactive state. It provided hope that this concept can finally produce the highly coveted drug against a mutant Ras. Furthermore, it showed that a drug that interacts with the inactive Ras state can work (Khan et al., 2020a). Continued efforts led to ARS-1620 (Janes et al., 2018) (Figure 2), with higher reaction kinetics and more potent drug action, inhibiting interaction with Raf and, consequently, MAPK signaling. It resulted in several lead compounds by pharmaceutical companies with subsequent clinical trials as described above.

Covalent drugs, especially allosteric ones, are powerful. However, to date their applicability has been limited to few cancers, particularly small cell lung cancer where the G12C mutation is abundant (Gorfe and Cho, 2019; Stephen et al., 2014). The account and promise shown by the impressive optimization of MRTX849 (Figure 2) that targets K-Ras$^{G12C}$ emphasizes, however, the challenges in the development of covalent drugs in structure-based design to improve drug potency and pharmacological properties, reaching nanomolar potency and selectivity *in vivo* (Fell et al., 2020; Gabizon and London, 2020).

Kessler et al. (2019) have targeted a pocket in K-Ras4B previously considered "undruggable" due to its comparative shallowness and polarity, rather than hydrophobic, between Switch I and Switch II (SI/II pocket) (Cruz-Migoni et al., 2019; Maurer et al., 2012; Quevedo et al., 2018; Sun et al., 2012). Starting from very weakly binding fragments and structure-based drug design, they discovered BI-2852 (compound 1) (Figure 3, upper left panel), a nanomolar inhibitor to this pocket distinct from covalent K-Ras$^{G12C}$ inhibitors. The pocket it binds differs from that of covalent K-Ras$^{G12C}$ inhibitors, which bind to the SII-P pocket in the Switch II region and is present in both the active (GTP-bound) and inactive (GDP-bound) forms of Ras proteins. It modulates MAPK signaling (pERK phosphorylation) and PI3K/AKT (pAKT) and inhibits *KRAS* mutant cell proliferation, suggesting that this pocket can be druggable.

Tran et al. (2020) proposed an alternative interpretation to the observation by Kessler et al. (2019) of BI-2852 inhibition at nanomolar affinity. Their analysis of the crystal structure of K-Ras4B with BI-2852, which included the neighboring unit cell, observed that BI-2852 induces a K-Ras4B dimer consisting of four molecules (two of BI-2852 and two of K-Ras4B) with rotational symmetry (Figure 3, right panel). This was confirmed experimentally by size-exclusion column chromatography at a concentration 1,000 times higher (3 mM) than those in which the cellular effects of BI-2852 were observed (Kessler et al., 2020), mass spectrometry, measurement of binding affinity (isothermal titration calorimetry), and surface plasmon resonance. Analysis of the crystal structure (PDB: 6GJ8) indicated that the pocket is formed by two K-Ras4B molecules where one copy of BI-2852 binds, suggesting an asymmetry, with one K-Ras4B presenting a larger pocket (Switch I/II pocket) and the other a smaller one. The second BI-2852 indicates symmetric formation. The complex is stabilized by four salt bridges altogether, pointing to the discovery by Kessler et al. (2019) of formation of a nonfunctional dimer stabilized by two molecules of BI-2852.

To further explore stabilization of nonfunctional K-Ras4B dimers for *KRAS*-driven cancers, Kessler et al. (2020) synthesized other possible dimeric Switch I/II-pocket binders. They discovered compound **2**, which dimerizes K-Ras4B with a $K_D$ of 3.8 μM. Soaking active K-Ras4B$^{G12D}$ (Bergner et al., 2019), they obtained K-Ras4B crystal structures of dimers with this compound at 1.9-Å resolution. Subsequent co-crystallization with compound **2** obtained a dimeric structure (at 1.57-Å resolution), a similar β-sandwich interface as with BI-2852, that resembles the one previously postulated by modeling and MD simulations of K-Ras4B molecules (Jang et al., 2016b). The

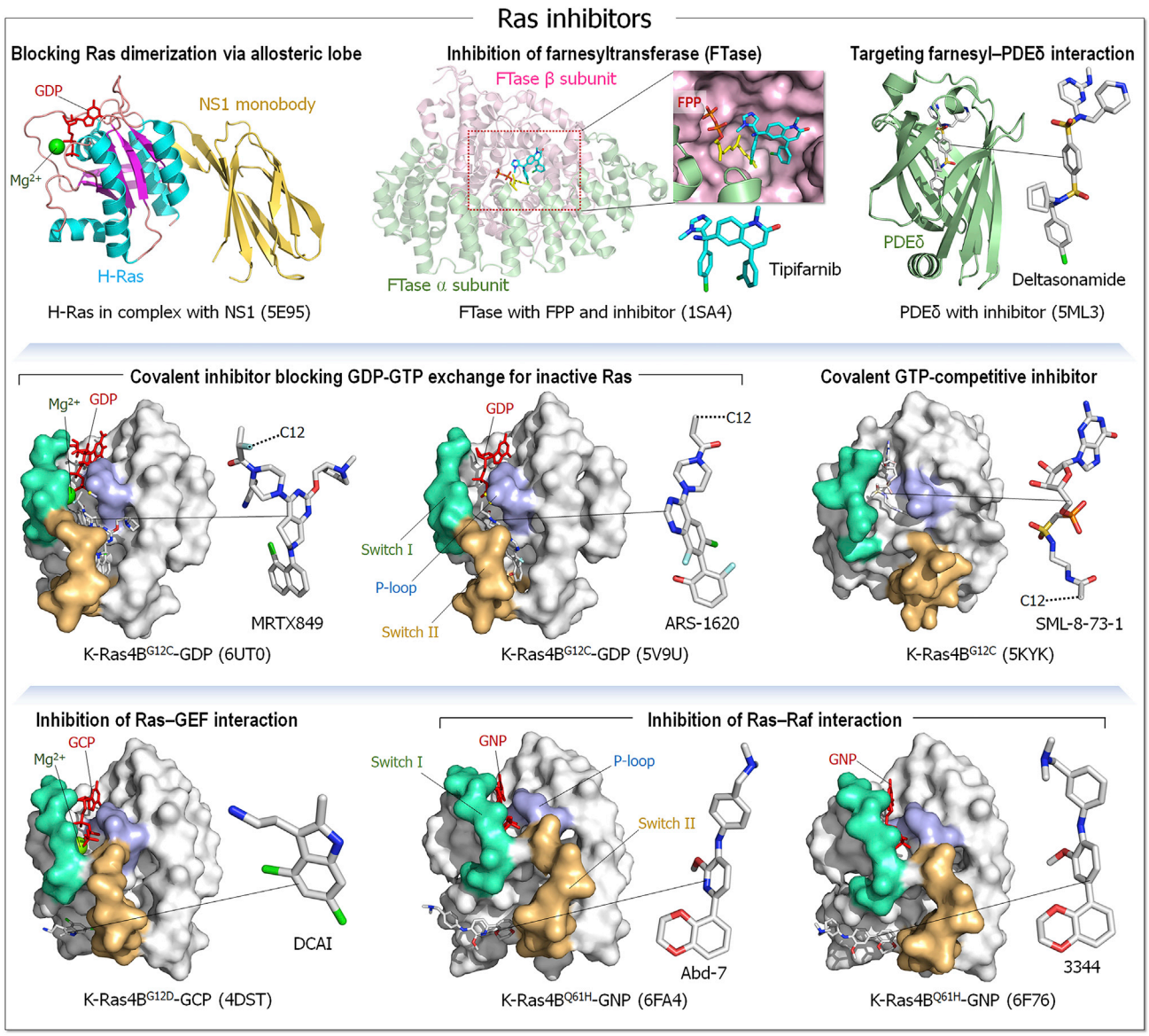



**Figure 2. Examples of Ras Inhibitors**

The first row shows crystal structures of the GDP-bound H-Ras in complex with NS1 monobody (PDB: 5E95), farnesyltransferase (FTase) containing the α and β subunits in complex with farnesyl diphosphate (FPP) and the inhibitor tipifarnib (R115777) (PDB: 1SA4), and phosphodiesterase δ subunit (PDEδ) inhibited by deltasonamide (PDB: 5ML3). Covalent drugs to the G12C mutation are shown in the second row; crystal structures of the GDP-bound K-Ras4B$^{G12C}$ with MRTX849 (PDB: 6UT0), the GDP-bound K-Ras4B$^{G12C}$ with ARS-1620 (PDB: 5V9U), and K-Ras4B$^{G12C}$ with the covalent GTP-competitive inhibitor SML-8-73-1 (PDB: 5KYK). The third row shows crystal structures of active Ras inhibited by noncovalent, small molecular drugs for the GCP-bound K-Ras4B$^{G12D}$ with DCAI (PDB: 4DST), the GNP-bound K-Ras4B$^{Q61H}$ with Abd-7 (PDB: 6FA4), and the GNP-bound K-Ras4B$^{Q61H}$ with 3344 (Abd-8) (PDB: 6F76).

modeled structure dimerizes through the effector lobe β-sandwich interface involving side-chain interactions of β1, β2, and β3 strands (Jang et al., 2016b) (Figure 3, lower left panel). The β-sandwich interface emerged from the exact β-sheet alignment due to hydrogen bond (H-bond) mismatch between the β2 strands. Especially noteworthy is that this dimeric interface was a minor dimeric form in the simulations, that is, with a smaller population, suggesting that compound **2** selects a minor species and stabilizes it.

Thus, whether monomers or dimers, allosteric interaction with a nonfunctional state will shift the ensemble away from the active state. Interestingly, the inactive K-Ras4B dimer that dimerizes through the effector lobe interface favors a shifted β-sheet extension through intermolecular H-bonds between the β2 strands formed by Glu37, Ser39, and Arg41 (Jang et al., 2016b). However, Raf easily competes with Ras molecules assembled through the effector lobe dimer interface due to the high-affinity interaction of RBD with the Ras effector binding site. The active K-Ras4B dimer, where the monomers interact through their allosteric lobes, favors an interaction through the α3-α4 helices, while H-Ras allosteric lobe dimerization favors the α4-α5 helical interface (Jang et al., 2016b, 2020).

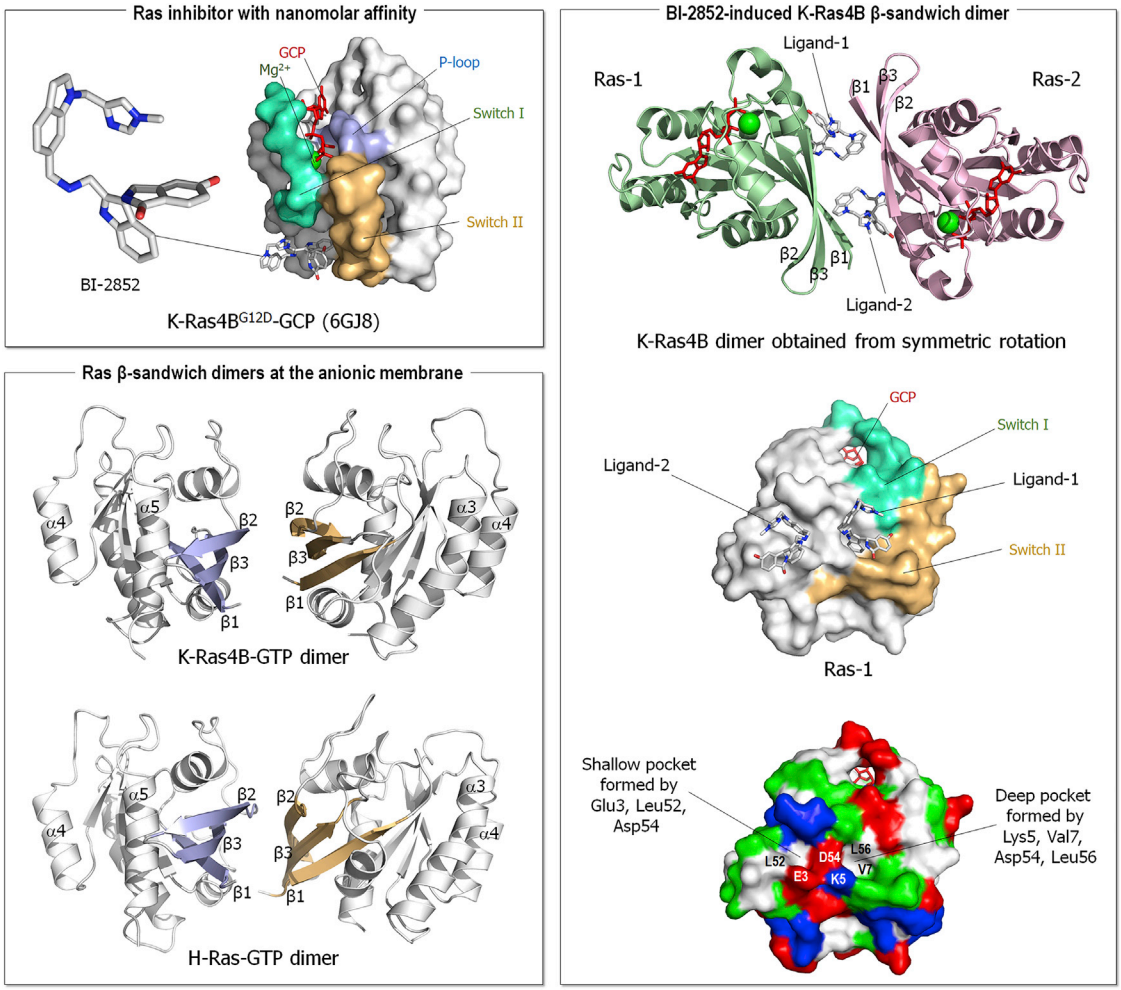



**Figure 3. Ras Inhibitor with Nanomolar Affinity**

Crystal structure of the GCP-bound K-Ras4B$^{G12D}$ with BI-2852 (PDB: 6GJ8) (upper left panel). The compound 1 drug, BI-2852, exhibits nanomolar affinity to a pocket between Switch I and Switch II. The BI-2852-induced Ras dimerization (right panel). Symmetric rotation of the K-Ras4B crystal structure reveals a β-sandwich dimer aligning its β1, β2, and β3 strands by two BI-2852 molecules. The first BI-2852 (ligand-1) binds the Switch I and II pocket, the deep pocket formed by Lys5, Val7, Asp54, and Leu56 on the first Ras (Ras-1). The second BI-2852 (ligand-2), a copy of the first, binds the shallow pocket formed by Glu3, Leu52, and Asp54 on Ras-1. The complex is stabilized by several salt bridges. The molecular dynamics (MD) simulations of Ras molecules at the anionic membrane (Jang et al., 2016b) previously reported the nonfunctional β-sandwich dimer for K-Ras4B-GTP and H-Ras-GTP (lower left panel).

Small molecules have also been developed to inhibit Ral GTPases, which are Ras GTPases identical in structure to K-Ras. The innovative strategy employed there similarly proposed to target GDP- rather than GTP-bound Ral, exploiting a new pocket (Yan et al., 2014). This pocket, which was not targeted before, harbors the K-Ras$^{G12C}$ mutation. This work also led to a follow-up discovery in Meroueh's lab of a covalent inhibitor that forms a bond with a tyrosine on Ral and Ras GTPases (Bum-Erdene et al., 2020), raising the possibility that covalent tyrosine linkage can open another venue in targeting of the Ras superfamily.

Drugging small GTPase Rheb is similarly challenging. Rheb activates the mechanistic target of rapamycin complex 1 (mTORC1), a master regulator of cellular growth and metabolism in the Ras/PI3K/AKT/mTOR pathway. Small molecule NR1 binds Rheb in the Switch II domain and selectively blocks mTORC1 signaling, but not mTORC2 (Mahoney et al., 2018).

Allosteric drugs are highly specific with fewer side effects and can tune the inhibition. Nonetheless, they still need to stably bind the protein, requiring some pockets. Covalent drugs also require a sufficiently reactive warhead, though not too reactive so as to avoid toxicity. The pocket should permit specific interactions that allow such reduction in warhead strength.

Finally, K-Ras$^{G12C}$ inhibitors target a somatic cysteine mutation. Since all cells are targeted, concerns about their likely toxicity persist (Sheridan, 2020). Selectivity against the wild type (therapeutic index) also depends upon the oncogene specificity in addition to the selectivity.

## SIGNIFICANCE

**The mechanism of action of many drugs is known. Prior knowledge in the development stage of the mechanism of action—at the molecular level—of effective drugs for the**

**targeted protein is enormously advantageous and reduces late-stage failures. Here we interrogate distinct mechanisms embodied by allosteric drugs for two classes of major drug targets: Ras superfamily and kinases. Among the emerging allosteric strategies for Ras is targeting its nonfunctional state—which differs from the allosteric drug strategies for kinases. In kinases, allosteric drugs mostly target the *active*, functional state. We ask why drugging the inactive K-Ras4B$^{G12C}$ can work but drugging the inactive kinase will likely not work. We propose that the reason is their distinct oncogenic mechanisms. In kinases, the mechanism of oncogenic kinase mutations involves increasing the population of the active conformations as compared with the inactive conformations. By contrast, Ras oncogenic mutations work by quenching the deactivation mechanisms, GTP hydrolysis, and nucleotide exchange. Thus, covalent allosteric inhibitors that bind to the inactive GDP-bound K-Ras4B$^{G12C}$ conformation can work—which is not the case in kinases, whose allosteric inhibitors work by altering the active-site conformation.**

**Drug discovery-wise the significance of our premise here is 3-fold: (1) it postulates that the mechanism of drug action relates to the mechanism of the oncogenic driver mutations; not to whether the protein is in the active or inactive state. (2) However, if drug binding allosterically blocks the target functional site, as in the case of Raf dimerization, it will work also for the inactive kinase states, and the GTP-bound Ras. That is, the location of the drug-binding site matters. (3) From the translational standpoint, this mechanism of action classification accelerates pharmaceutical development. It postulates that *drug action is related to blocking the mechanism of activation—not to the active or inactive state*, extending drug classification charts.**

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## AUTHOR CONTRIBUTIONS

R.N. and H.J. designed research. R.N. performed research and prepared the initial draft. H.J. generated the figures. R.N. and H.J. wrote and edited the paper. R.N., H.J., A.G., O.K., and V.G. have given approval to the final version of the manuscript.

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