HHS Public Access

Author manuscript

Mini Rev Med Chem. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:

Mini Rev Med Chem. 2016 ; 16(5): 358–369.

How to Target Activated Ras Proteins: Direct Inhibition *vs*. Induced Mislocalization

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Abstract

Oncogenic Ras proteins are a driving force in a significant set of human cancers and wild-type, unmutated Ras proteins likely contribute to the malignant phenotype of many more. The overall challenge of targeting activated Ras proteins has great promise to treat cancer, but this goal has yet to be achieved. Significant efforts and resources have been committed to inhibiting Ras, but these energies have so far made little impact in the clinic. Direct attempts to target activated Ras proteins have faced many obstacles, including the fundamental nature of the gain-of-function oncogenic activity being produced by a loss-of-function at the biochemical level. Nevertheless, there has been very promising recent pre-clinical progress. The major strategy that has so far reached the clinic aimed to inhibit activated Ras indirectly through blocking its post-translational modification and inducing its mislocalization. While these efforts to indirectly target Ras through inhibition of farnesyl transferase (FTase) were rationally designed, this strategy suffered from insufficient attention to the distinctions between the isoforms of Ras. This led to subsequent failures in large-scale clinical trials targeting K-Ras driven lung, colon, and pancreatic cancers. Despite these setbacks, efforts to indirectly target activated Ras through inducing its mislocalization have persisted. It is plausible that FTase inhibitors may still have some utility in the clinic, perhaps in combination with statins or other agents. Alternative approaches for inducing mislocalization of Ras through disruption of its palmitoylation cycle or interaction with chaperone proteins are in early stages of development.

Keywords

H-Ras; K-Ras; lovastatin; N-Ras; prenylation inhibitors; statins; subcellular localization

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CONFLICT OF INTEREST

The authors have no conflicts of interest. E.B. has been supported by the Wayne State University School of Medicine, the cancer biology graduate program, and the training program in the biology of cancer [T32-CA009531]. Relevant work in J.J.R.'s laboratory has been supported by Department of Army [W81XWH-13-1-0097], and the Developmental Therapeutics Program, Karmanos Cancer Institute. Relevant work in R.R.M.'s laboratory has been supported by the National Cancer Institute [R01 CA81150 and CA131990], the Department of the Army [NF99035, NF020054, NF043107, and NF093146], by NTAP (Neurofibromatosis Therapeutic Acceleration Program), and by philanthropic support from Dan and Jennifer Gilbert. The content of the information does not necessarily reflect the position or policy of the U.S. government, and no official endorsement should be inferred.

1. INTRODUCTION

Mutated Ras proteins play critical roles in both the development [1] and maintenance of tumors [2, 3]. In contrast to the increasingly successful therapeutic targeting of other driving oncogenes to treat human cancer [4], Ras has yet to be effectively blocked in the clinic [5]. This void is particularly serious because Ras mutations are the most prevalent oncogenic events in human cancer. Although previous estimates of 30% incidence of Ras mutations in human cancers are likely high due to selection bias for certain tumor types with particularly high rates, an average pan-Ras mutation incidence of 16% points to the potential that would result if effective targeting could be achieved [6]. Positive Ras mutation status is not only a major driver of disease, it also correlates with poor prognosis and resistance to therapy [7], for example in colorectal carcinoma and lung adenocarcinoma [8, 9].

Ras proteins are the products of genes that were the first identified human oncogenes [10]. There are three human Ras genes that code for four distinct proteins: H-Ras; N-Ras; and two variants of K-Ras produced by alternative splicing, K-Ras4A and K-Ras4B. We are still discovering fundamental new information about these isoforms. For example, K-Ras4A used to be viewed as a minor variant and was less studied, recent application of isoform-specific PCR and antibodies has shown expression at significant levels in all human cancer cell lines and tissue specimens tested [11]. This result is particularly notable in light of previous evidence that K-Ras4A may have an important role in lung carcinoma [12]. While the Ras proteins all have identical effector domains, studies have shown that these isoforms have some distinct functions [13–15]. For example, K-Ras is the only form that binds to calmodulin [16], that can confer stem-like properties to certain cell types [7], and that has essential functions in mouse embryogenesis [17, 18]. Insertion of H-Ras at the K-Ras locus allows embryonic development, but then reveals a late-onset cardiomyopathy [19].

In regard to the pathologic expression of each isoform, K-Ras is mutated most often, particularly in pancreatic, intestinal, cholangio, and lung carcinomas, while N-Ras is mutated more in certain skin and hematopoietic cancers. H-Ras mutations are less common, but occur more in salivary gland and urinary tract cancers [6]. Further examination of the mouse model that has H-Ras inserted at the K-Ras locus shows that carcinogen-induced pulmonary tumorigenesis is maintained at a similar rate, with the lung cancers now driven by oncogenic H-Ras [12]. This spectrum of results suggests that the pattern, timing and level of expression of the Ras protein, rather than the specific isoform, may be more crucial for some aspects of development and tumorigenesis. Nevertheless, there are also some isoform-specific functions. Such discrepancies between isoforms are generally ascribed to differences in their short hypervariable regions (which are located just before the C-termini) and associated with their distinct subcellular locales [20–22].

In addition to the driving roles played by oncogenically-mutated Ras, it is likely that unmutated Ras proteins may also contribute to human cancers. For example, oncogenic K-Ras activity may require functional H-Ras or N-Ras to drive its effects [23, 24]. Further, activated wild-type Ras proteins may also promote cancers that do not harbor mutated Ras. For example, in breast cancer Ras mutations are rare, but there is strong evidence that increased growth factor and HER2 signaling induce over-activated Ras proteins to produce

the transformed phenotype [25–27]. In type 1 neurofibromatosis (NF1), the loss of expression of neurofibromin, which normally functions to deactivate Ras, provides a route for over-activated Ras proteins to drive tumor formation [28–30]. Sporadic loss of neurofibromin expression likely drives Ras activation in other cancers, such as melanoma and lung adenocarcinoma, where it has also been linked to treatment resistance [31, 32].

Ras proteins drive pathways that can regulate perhaps all of the characterized hallmarks of cancer [33]. Thus, effective blockade of activated Ras could lead to beneficial outcomes if it could be achieved with acceptable levels of toxicity. The current status of several potential approaches are discussed below, with reference to the complexity of the Ras isoforms, and the degree to which there is a need to consider both the mutated and wild-type proteins. The focus will be on the status of attempts to directly target the protein, and to indirectly target it through induced mislocalization. The ongoing efforts to target pathways downstream of activated Ras, and that are beginning to prove useful in cancer treatment [34], have been reviewed elsewhere [5, 35].

1.1. Direct Targeting of Ras

There are several barriers to direct therapeutic targeting of activated Ras. The first problem is that Ras is a small GTPase that is regulated through a cycle of GTP binding for activation (which is stimulated by guanine nucleotide exchange factors or GEFs) and then GTP hydrolysis to GDP for deactivation (which is stimulated by GTPase-activating proteins, or GAPs) [36], see (Fig. 1). Thus the conventional understanding of direct inhibition of an enzyme, i.e., to block its enzymatic activity, would actually produce an increase in the GTP-bound, activated fraction of Ras and so be the opposite of the mechanistic goal. Structural analyses of Ras indicated that it was unlikely that a small molecule could be designed to restore to oncogenic variants the lost GTPase activity and sensitivity to GAPs [37]. Furthermore, screening efforts to discover such compounds failed [7]. A related problem underlies the challenge of targeting Ras for treatment of NF1 [38]. The Ras in this case is wild-type and so maintains its endogenous GTPase activity, with the over-activation caused by loss of expression of the GAP, neurofibromin [28, 29]. There is no clear route to design a small molecule that could replace the lost expression and activity of neurofibromin.

The second problem is that Ras binds GTP with picomolar affinity, and so it has not generally been feasible to design a small molecule that can displace the activating nucleotide [39]. This situation differs significantly from protein kinases, where ATP binding typically occurs with micromolar affinity. As a result, small molecule nucleotide analogues (typically with nanomolar affinities) effectively block ATP binding to kinases, but are unable to disrupt GTP binding to Ras [40]. Recent publications involving the GDP analog named SML-8-73-1 by Gray, Westover and colleagues provide the first example of success in this area [41, 42]. These groups, and that of Shokat [43], have addressed an interesting premise: why not target the activating, mis-sense substitution itself? Such an approach would have elegant selectivity for the pathological driver. Their efforts have produced small molecules that target K-Ras with the G12C mutation, which commonly occurs in tobacco-induced lung cancer [44]. The feasibility of such covalent targeting is supported by recent studies with ibrutinib, which binds a cysteine in the active site to inhibit Bruton’s tyrosine kinase. Although the

compounds targeting Ras have only been shown to act *in vitro* so far, the approval of ibrutinib for treatment of relapsed mantle cell lymphoma provides a paradigm for this approach [45]. Shokat and colleagues developed a set of small molecules that could irreversibly bind to K-Ras G12C and prevent mutant protein—but not wild-type—from entering the GTP-bound state [43]. In parallel efforts, Gray and Westover and colleagues identified a GDP analog (SML-8-73-1) and a prodrug derivative (SML-10-70-1) that had the ability to covalently bind and specifically inactivate K-Ras G12C by leaving it in an open conformation that cannot interact productively with effectors [41, 42]. Although the compounds will require significant further pre-clinical optimization [46], these developments have rejuvenated interest in directly targeting Ras.

The third problem is that the function of activated Ras-GTP is transmitted through its formation of complexes with effectors [47], and small molecule inhibition of such protein:protein contacts has often proved difficult [48]. The structure of Ras does not have any clearly exploitable pockets to target, and allosteric regulation sites have not been revealed [43, 49]. A proof-of-principle study used expression of a blocking antibody fragment to demonstrate that oncogenic function of mutated K-Ras could be inhibited in a mouse model [50]. These results are a successor to earlier studies in which micro-injection of Ras antibodies into fibroblasts demonstrated the essential role of proto-oncogenic Ras function in serum stimulation of G_1-to-S phase progression [51]. Recently, Kataoka and colleagues demonstrated that binding of H-Ras.GTP to c-Raf1 could be inhibited by small molecules both *in vitro* and *in vivo*. These inhibitors were also capable of down-regulating a number of Ras-driven pathways, and were orally active against a K-Ras driven colon cancer xenograft [52].

Another potential mechanism to inhibit Ras activation would be to block its activation by a GEF. Progress has recently been made in this area by disruption of Ras interaction with Son of Sevenless (Sos) [49, 53, 54]. On the other hand, the ultimate utility of targeting the GEF step has not yet been determined. Given that Ras mutations favor constitutive signaling, it seems likely that wild-type proteins would be significantly more dependent on GEF interaction, i.e., there is probably differential reliance on basal *vs*. stimulated nucleotide exchange [24, 54, 55]. Thus it is plausible that disrupting Sos activation of normal Ras could lead to unwanted toxicity if oncogenic Ras is the desired target.

The degree to which inhibitors of Sos/Ras interaction will also block Ras activation by other GEFs has generally not been defined. Sos is likely the principal activating pathway for Ras as a driver of cellular proliferation [56] and thus the logical target for these efforts. If compounds also block other GEFs that activate Ras in different contexts, then adverse events could occur. For example, Ras-GRP GEFs play critical roles in regulation of Ras in lymphocytes to control immune functions [57]. In T cells, subtle regulation of Ras determines development and selection, and small perturbations in Ras activation could lead to auto-immune dysfunction [57–59]. Another important example is provided by Ras-GRF GEFs that regulate neuronal Ras, particularly the H-Ras isoform [60], to control synaptic plasticity [61, 62]. Ras-GRF control of Ras is likely to integrate multiple neurotransmitter and neurotrophic pathways [63–67]. Disruption of Ras activation in the brain is strongly

linked to disordered cognition and mental retardation [68]. It is clear that the accurate regulation of Ras is vital for normal functioning.

1.2. Indirect Targeting of Activated Ras Through Inhibition of Prenylation to Cause Mislocalization

Ras proteins require a sequence of post-translational modifications to appropriately localize to membranes and become functionally active [69–71], see (Fig. 2). The demonstration that correct localization was essential for Ras's transformative activity was the basis for a strategy to block Ras-driven cancers by inhibiting these modifications [72, 73]. In addition, selective toxicity might occur if mislocalized, constitutively-active Ras brought related signaling molecules to an abnormal environment [72, 74]. The initial step in the series that leads to membrane association and activity is the post-translational prenylation of the cysteine residue in the CaaX sequence (where C is cysteine, "a" is any residue (typically aliphatic), and X is glutamine, methionine, serine or leucine) found at the carboxyl terminus of Ras. Prenylation entails the covalent attachment of farnesyl or geranylgeranyl moieties to the cysteine catalyzed by farnesyl transferase (FTase) or geranylgeranyl transferase-I (GGTase-I), respectively [75]. Which moiety is attached depends on the CaaX sequence, and if a farnesyltransferase inhibitor (FTI) is present. All Ras molecules preferably undergo farnesylation. However, if an FTI is present, then K- and N-Ras, because of a leucine at the "X" position, can undergo geranylgeranylation by GGTase-I [76–78].

1.2.1. Inhibition of Prenylation by Suppressing Isoprenoid Synthesis—A popular strategy for disrupting Ras localization targeted prenylation. One general approach targets isoprenoid biosynthesis. Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are co-substrates in FTase and GGTase-I reactions respectively, are intermediates in the cholesterol biosynthetic pathway [73], see (Fig. 2). Presumably, limiting the supply of precursors needed for FPP and GGPP synthesis should reduce prenylation. Early studies focused on the use of statins (e.g., particularly lovastatin) to inhibit HMG-CoA reductase. Preclinical studies with lovastatin indicated that it could inhibit prenylation in cultured cells. Furthermore, it showed promising therapeutic effects in murine tumor xenograft studies and, in some cases, the therapeutic effect correlated with reductions in the tumors' prenylated Ras content [79, 80]. However, the concentrations needed to affect Ras prenylation in these preclinical studies are unattainable in humans because of dose limiting toxicity [81]. Specifically, although a lovastatin plasma level of 3.9 μM was achieved in a phase I clinical trial, it could not be maintained because of toxicity [82]. Treatment of cultured vascular smooth muscle cells with 3 μM lovastatin was sufficient to greatly impair their proliferation and reduced prenylation of RhoB, but had negligible effect on Ras [83]. The upper range of tolerable lovastatin dosing in humans results in plasma levels of ~225 nM and does not affect Ras processing in peripheral blood cells [84]. Irrespective of this limitation, statins continue to be widely used in preclinical studies, especially in combinational protocols, at concentrations ≥ 10 μM. Although sufficient to modulate Ras prenylation, such concentrations are physiologically irrelevant since they cannot be achieved in humans. There have also been recent clinical trials with statins with the goal of improving cognitive functions in children and adults with NF1. Despite the previous results in humans, the rationale for these studies is that there will be an inhibition

of Ras [85–87]. The largest trial, with 84 children and a placebo-controlled, double-blind design, failed to demonstrate any beneficial effects of simvastatin treatment [87].

Nitrogen-containing bisphosphonates (nBPs) are synthetic analogues of inorganic pyrophosphate. They readily bind Ca^{2+} and have a very high affinity for areas of bone undergoing osteoclastic resorption [88–90]. Because of this affinity, and their cytotoxicity towards osteoclasts, nBPs have been used in the treatment of osteoporosis, Paget's disease, and metastatic bone disease [90, 91]. Like statins, nBPs suppress isoprenoid synthesis, but do so by inhibiting farnesyl pyrophosphate synthase (FPP synthase), the enzyme immediately responsible for the generation of FPP, which is the precursor to GGPP [89, 92], see (Fig. 2). *In vitro* studies, with a variety of cell types, demonstrated that nBPs suppress the conversion of [^{14}C]mevalonate into [^{14}C]FPP and [^{14}C]GGPP [89, 93], reduce the prenylation of Ras [93] and Rap1A [89], and cause a loss of membrane-associated Ras [94]. In addition to their proven effectiveness in the treatment of a variety of osteoclast-mediated bone conditions, mouse xenograft studies suggest that nBPs may be useful in the treatment of some non-bone-related cancers [89, 95]. At issue is whether these latter *in vivo* anti-cancer effects are mediated by protein deprenylation.

An alternative approach for modifying production of isoprenoids entails the targeted inactivation of geranylgeranyl diphosphate synthase (GGDPS), a cytosolic enzyme responsible for the conversion of FPP to GGPP [95]. A variety of isoprenoid bisphosphonates have been synthesized that selectively inhibit the *in vitro* activity of purified GGDPS with high nM to low micromolar potency [95–97], see (Fig. 2). Cell culture studies confirmed that the more potent of these also suppressed the prenylation of Rap1A (a GGTase-I substrate) and Rab6 (a GGTase-II substrate) to a level comparable to 10 μM lovastatin [96, 97]. However, unlike lovastatin, the GGDPS inhibitors did not affect the prenylation of Ras [96, 97]. Furthermore, it has been reported that cotreatment of cultured K562 leukemia cells with lovastatin and the GGDPS inhibitor digeranyl bisphosphonate resulted in a synergistic suppression of both Rap1a and Rab6 prenylation, but an antagonism of lovastatin's inhibitory effects on Ras prenylation [96]. This is not surprising since inhibition of GGDPS activity would lead to a build up of FPP, and thus favor the farnesylation of Ras. Interestingly, concentrations of the GGDPS inhibitor digeranyl bisphosphonate sufficient to inhibit prenylation in cultured K562 cells also suppressed cell growth and induced apoptosis [96]. Furthermore, the anti-proliferative and pro-apoptotic activities of digeranyl bisphosphonate were synergistically enhanced by co-treatment with lovastatin [96]. These latter findings suggest that prenylated proteins other than Ras may be the targets and basis for the anti-proliferative/pro-apoptotic activities of some prenylation inhibitors.

1.2.2. Inhibitors of Prenylation Enzymes—A second general approach for modulating Ras prenylation involves suppression of FTase or GGTase-I catalytic activities. This entails the use of agents that suppress prenylation by competing with FPP, GGPP, or CaaX containing protein substrates for binding to FTase or GGTase-I, see (Fig. 2). Early attempts focused on the development of FTIs, and an extensive preclinical literature accumulated indicating that FTIs were effective at reversing H-Ras-mediated transformation, and suppressing H-Ras driven tumor xenografts in mice [98–100]. This strategy became the

dominant one for targeting oncogenic Ras [101]. Ultimately, six different FTIs entered clinical trials [i.e., tipifarnib (aka R115777 or Zarnestra), lonafarnib (aka SCH66336 or Sarasar), BMS-214662, L778123, L744832 and FTI-277]. Of the six, the CaaX box competitive inhibitor tipifarnib advanced the farthest and was tested in stage III trials involving colorectal and pancreatic cancers [102, 103]. The results of these stage III tipifarnib studies were very disappointing. No significant anti-tumor activities were observed. In retrospect, an obvious explanation for the failure of these trials was the focus on malignancies driven by activated K-Ras, which are able to undergo alternative geranylgeranylation in the presence of an FTI [76–78].

Although a logical explanation exists for the negative outcomes of the tipifarnib phase III trials, the lack of efficacy branded FTIs as being failed drugs for the treatment of human cancer. Unfortunately that perception has persisted in spite of more recent studies indicating that FTIs may be useful in the treatment of astrocytomas, gliomas, and a subset of patients with hematological cancers [104–106]. It is not clear whether inhibition of Ras prenylation underlies any of these responses, however. Lonafarnib, which also advanced as far as phase II/III cancer trials, has more recently shown great promise as a novel therapy for progeria [107]. In this case the rationale is to block farnesylation of a variant of lamin-A, not Ras. It is, however, still plausible that FTIs may be effective against cancers that are driven by oncogenic H-Ras, which would not be able to escape inhibition through undergoing alternative prenylation [see [5] for review]. Mutated H-Ras is present in approximately 3% of human cancers [35]. Tumor profiling is already identifying patients with oncogenic H-Ras [108]. Perhaps by supporting such results with bioinformatic analysis to delineate driving pathways [109], it might be possible to select a patient population who may be primed to respond in a FTI clinical trial.

Both pharmacological and molecular approaches have documented the contributions of GGTase-I to Ras prenylation, so it is reasonable to ask whether it would be possible to achieve effective inhibition of K-Ras prenylation and localization by the combined inhibition of both FTase and GGTase-I. For example, K-Ras prenylation is maintained in cells/tissues derived from mice deficient in either FTase or GGTase-I due to a conditional knock out of the b-subunit of either enzyme [110]. However, dual deficiency in FTase and GGTase-1 reduces K-Ras prenylation in both cell lines and tissues derived from knockout mice [110]. Similarly, cotreatment with FTI and GGTase-I inhibitors (GGTIs) facilitates the deprenylation of both N- and K-Ras in cultured cells and murine xenograft models [99, 111, 112]. However, doses of GGTIs sufficient to block K-Ras prenylation *in vivo* were observed to be toxic, and lethal if chronically administered [111]. Furthermore, the anti-tumor activity of GGTIs towards models driven by activated K-Ras has been reported to be independent of K-Ras prenylation status [99, 113].

In addition to combinations of molecules that selectively target FTase or GGTase-I, considerable effort has gone into the synthesis of dual prenyltransferase inhibitors (DPIs), see (Fig. 2). As nicely documented in a paper by Tucker *et al.*, the ratio of FTI to GGTI activity can vary markedly from DPI to DPI [114]. Of the many DPIs synthesized, L-778,123 is probably the most extensively examined. Low micromolar concentrations of L-778,123 were reported to suppress the prenylation of H-, N- and K-Ras in HL-60

leukemia cells [115]. However, doses of L-778,123 sufficient to inhibit the prenylation of Rab6 and HDJ2 in the peripheral blood mononuclear cells (PBMCs) of treated dogs had no effect on canine K-Ras prenylation [113]. Similarly, two phase I pre-clinical studies with L-778,123 [113, 116] reported that doses and scheduling protocols having no, or acceptable side effects, partially suppressed patient PBMC Rab6 and HDJ2 prenylation. However, no deprenylation of K-Ras occurred, even at doses that had to be discontinued because of adverse side effects. In the clinical studies the level of suppression of HDJ2 and Rab6 deprenylation correlated with plasma L-778,123 concentrations.

1.2.3. Combinations of Statins and Inhibitors of Prenylation Enzymes—A third general approach for modulating Ras prenylation entails treatment with a statin plus FTI or GGTI. The statin dosage in such protocols is insufficient by itself to inhibit prenylation, but sufficient to decrease the isoprenoid pool available for FPP and GGPP synthesis. Several studies document the synergistic anti-proliferative/cytotoxic effects of statin and FTI cotreatment on H-, K- and N-Ras driven tumors. For example, Ding *et al.* reported that a combination of atorvastatin and tipifarnib inhibited the growth of K-Ras mutated pancreatic cancer cells and xenografts in excess of the additive inhibitory effects of the two agents [117]. Unfortunately, there was no determination of whether the observed effect correlated with the deprenylation of Ras. Similarly, Morgan *et al.* reported that lovastatin synergized with the FTI L-744,832 to inhibit the *in vitro* growth of primary myeloma cells isolated from bone marrow aspirates of patients with multiple myeloma. In this study, the concentration of lovastatin (20 μM) used for analyses of Ras prenylation status was by itself sufficient to cause deprenylation of both N- and K-Ras [112]. In contrast, Yonemoto *et al.* reported that cotreatment of H-Ras transfected NIH3T3 fibroblasts with the FTI J-104,871 and 2.5 μM lovastatin shifted the IC_{50} for the FTI from 3.1 to 0.5 μM, and that this change was accompanied by a parallel deprenylation of H-Ras [118].

The strongest rationale for combinations of a statin with inhibitors of FTase or GGTase likely applies to FTIs or GGTIs that compete with the prenyl co-substrate of the enzyme rather than with the CaaX peptide substrate. In such situations, the limitation of the prenyl substrate pools imposed by the statin would most likely potentiate the action of the FTI or GGTI [83, 119–121]. For example, combinations of lovastatin and farnesyl pyrophosphate prodrug FTIs dramatically synergize with one another in their killing of NF1 malignant peripheral nerve sheath tumors [122–124]. Prenylation analyses in these studies indicated that the combinational treatment decreased the prenylation of not only Ras, but also of Rap1A and Rab5, proteins normally geranylgeranylated by GGTase-I and GGTase-II, respectively. Importantly, these cells express little or no H-Ras and the predominant activated isoform is N-Ras [125]. Further, the synergy occurred with concentrations of lovastatin as low as 100–250 nM, i.e., levels that are achievable and tolerated in humans.

1.2.4. Summary of Prenylation as a Target for Inhibition of Activated Ras—Studies of Ras prenylation and inhibitors have yielded several key insights. First, statins and prenylation inhibitors often exhibit therapeutic effectiveness against tumors that are either not Ras-driven, or, if they are Ras-driven, there is no change in Ras prenylation status

following inhibitor treatment [126–128]. Hence, in some cases, the anti-cancer activities of statins or FTIs and GGTIs appear to be independent of effects on Ras prenylation.

Second, the effects of prenylation inhibitors and high dose statin treatment are not limited to Ras. Hundreds of proteins are predicted to be prenylated [129]. It is likely that the deprenylation of some of these non-Ras proteins (e.g., RhoB, Rheb) contributes to the cytostatic/cytotoxic activities of statins and FTIs and GGTIs. It is also plausible, as discussed above, that endogenous H-Ras might be a critical target for FTI action [5].

Third, individual prenylated proteins differ in their sensitivities to FTIs and GGTIs. For example, K-Ras is less sensitive to deprenylation than is N-Ras [130]. Similarly, in a study in which K-Ras farnesylation could be investigated independent of any confounding geranylgeranylation, comparable deprenylation of K-Ras and NDJ2 (both of which are normally farnesylated) required a 10-fold higher concentration of the FTI tipifarnib to block K-Ras [111]. Such findings emphasize the limitations of using the deprenylation of marker proteins, such as Rap1A and NDJ2, as surrogates for Ras prenylation status. There is also the general problem of studying easily biopsied tissues in clinical studies, such as peripheral blood mononuclear cells, rather than the more difficult-to-biopsy tumor itself. Thus, it is often difficult to know in clinical studies if deprenylating agents actually affect Ras in the target tissue.

Fourth, the prenylation enzymes and the effects of many prenylation inhibitors need to be considered in terms of relative selectivity rather than specificity. For example, analyses of 23 CaaX box competitive FTIs indicated that 17 were also potent inhibitors of GGTase-II [131]. In the case of GGTase-I, it is well established that it can farnesylate prenyl acceptors that have a C-terminal leucine in their CaaX box motif [132, 133]. Similarly, *in vitro* studies indicate that FPP binds to GGTase-II, and that GGTase-II can farnesylate Rab7 if the ratio of FPP to GGPP is high [134]. This ability of GGTase-I and -II to catalyze farnesylation may provide an explanation as to why FTIs designed to compete with FPP might also inhibit the prenylation of proteins that are normally GGTase-I and -II substrates.

1.3. Alternative Approaches to Target Activated Ras by Preventing Ras Maturation and Localization

Ras prenylation is the first step in its process of maturation and localization. The sequence continues with proteolytic cleavage after the prenylated cysteine by Ras converting enzyme (Rce1), and subsequent methylation by isoprenylcysteine carboxyl methyltransferase (Icmt), see (Fig. 2). Both of these steps have been considered as potential therapeutic targets, though such work does not seem particularly promising. Likely problems include lack of specificity for Ras proteins over the many other substrates of these enzymes and consequent toxicity [see [5] for review].

Ubiquitination has also been implicated in controlling the cellular localization of Ras. In a series of papers, de la Vega and colleagues described the dynamic relationship between the de-ubiquitinase USP17 and Rce1 to control H- and N-Ras trafficking, but leaving K-Ras4B unaffected [135]. Briefly, ubiquitination of an Rce1 isoform localizes it to the endoplasmic reticulum (ER). In the presence of active USP17, deubiquitination occurs, causing this

isoform to leave the ER and be degraded. As a result of this degradation, CaaX modification is blocked [136]. Research in this area could lead to another potential alternative for targeting Ras.

Subsequent to the prenylation, proteolysis and methylation reactions, an additional palmitoylation step is required for membrane association of H-, N-, and K-Ras4A [137], see (Fig. 3). K-Ras4B and 4A have stretches of lysine residues near their C-termini that serve as polybasic signals for membrane association [71]; K-Ras4A achieves membrane association due to the effects of both its polybasic residues and palmitoylation [11, 138]. In addition to the isoform-specific differences, another distinction at this step is that palmitoylation is clearly reversible, with specific enzymes catalyzing both the addition and removal of the palmitoyl moiety, and that this reversibility governs the subcellular localization and function of H- and N-Ras [139–143]. There is increasing interest in whether these reactions may provide therapeutic targets to block activated Ras (but not K-Ras4B) function [144–147]. The work is at a comparatively early stage and the complexity of the system makes it hard to study [148], suggesting that there may be a number of unanticipated obstacles. Yet the complexity also gives several reasons for optimism. As we learn more about the palmitoylation/depalmitoylation cycle of Ras, regulatory mechanisms will be revealed [149]. It is plausible that such steps may ultimately be exploited as targets for intervention to block Ras function. One reason why effective therapeutic targeting of the prenylation, proteolytic and methylation steps has proven to be difficult is that there is generally a single enzyme for each reaction that has many substrates in addition to Ras. In contrast, the enzymology of palmitoylation is extremely complex with many identified genes [150, 151]. Early results suggested that H- and N-Ras palmitoylation was under control of specific enzymes [152]. For example, DHHC9, which can be inhibited by microRNA-134, may be predominantly responsible for palmitoylation of H-Ras in cortical neurons [153]. If a specific subset of palmitoylation/depalmitoylation enzymes with limited redundancy controls Ras, then some selective targeting might be achieved [154]. On the other hand, dynamic palmitoylation is required for synaptic remodeling [155], which, together with the evidence that H-Ras function is critical here [see [5] for review], could suggest that blocking these reactions may lead to cognitive problems.

The functions of the lipid modifications of Ras proteins are expressed through induced interactions with both the lipid bilayer and also targeting or chaperone proteins [156]. These interactions have also been developed as potential therapeutic targets for activated Ras, see (Fig. 3). One that has made it to the clinic is the compound salirasib, which is an analog of the farnesyl isoprenoid [157], and that may interfere with the chaperoning activity of galectins toward farnesylated Ras proteins to disrupt their localization and activity [158]. It is likely that interactions with other, non-Ras farnesylated proteins will also be disrupted. A related compound that has been tested in patients is TLN-4601 [159]. Neither drug has been demonstrated to affect K-Ras function in patients [159–161], although a decrease in total K-Ras protein was reported in paired (pre- and post-treatment) tumor biopsies in two patients taking salirasib [161]. In a recent trial on hematological malignancies, salirasib efficacy was modest and did not correlate with Ras mutation status [162]. Although the diseases under study would presumably have made biochemical examination of peripheral blood mononuclear cells relevant, results from such studies were not reported.

Another chaperone for farnesylated proteins, including Ras, is phosphodiesterase-delta (PDEδ) [163]. Recently Zimmerman and colleagues have described small molecules that disrupt the interaction of K-Ras and PDEδ. One of these, termed deltarasin, decreased K-Ras function in pancreatic cells and inhibited growth of xenografts [164, 165]. The degree to which there is a selective effect on Ras as opposed to other farnesylated proteins and whether the inhibition will be effective *in vivo* and exerted through block of K-Ras function remain to be defined.

CONCLUSION

When it was realized that oncogenic Ras mutants were a driving force in many human cancers, great efforts and resources were committed to targeting these proteins. Unfortunately, these energies have so far produced little impact in the clinic. To an extent, directly targeting activated Ras parallels the trials and tribulations faced by scientists trying to therapeutically exploit tumor suppressors like p53, in that the biochemical lesion in oncogenic Ras is actually a loss of GTPase function. While the efforts to indirectly target Ras through FTIs were rationally designed, this strategy suffered from insufficient attention to the distinctions between the isoforms and lack of consideration of the fundamental biology of Ras prenylation. This led to their subsequent failure in large-scale clinical trials targeting K-Ras driven lung, colon, and pancreatic cancers. Despite these setbacks, efforts to indirectly target activated Ras through inducing its mislocalization have persisted and progress continues.

Acknowledgments

The authors acknowledge their longstanding collaboration with the late Prof. R.A. Gibbs for the joint development of several of the ideas presented here.

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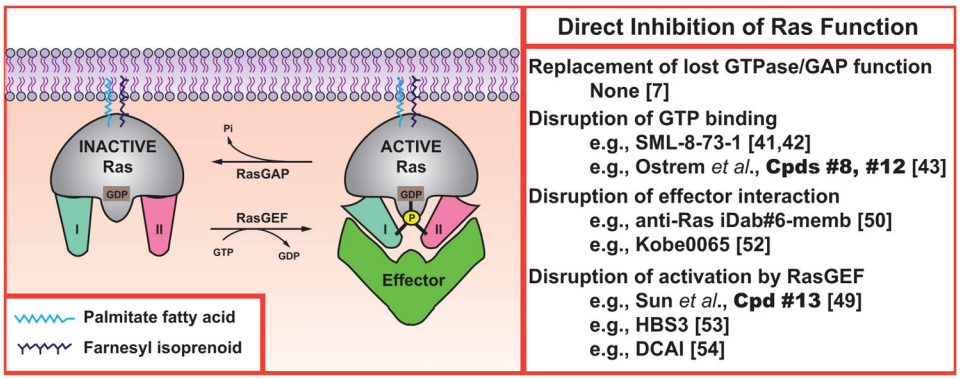

Fig. (1). Direct inhibition of Ras Function

The Ras activation/deactivation cycle and interaction with downstream effectors provides several potential therapeutic targets. Mature Ras proteins are anchored at the membrane and achieve an active conformation that interacts with effectors following the binding of GTP. This active conformation is lost upon GTP hydrolysis.

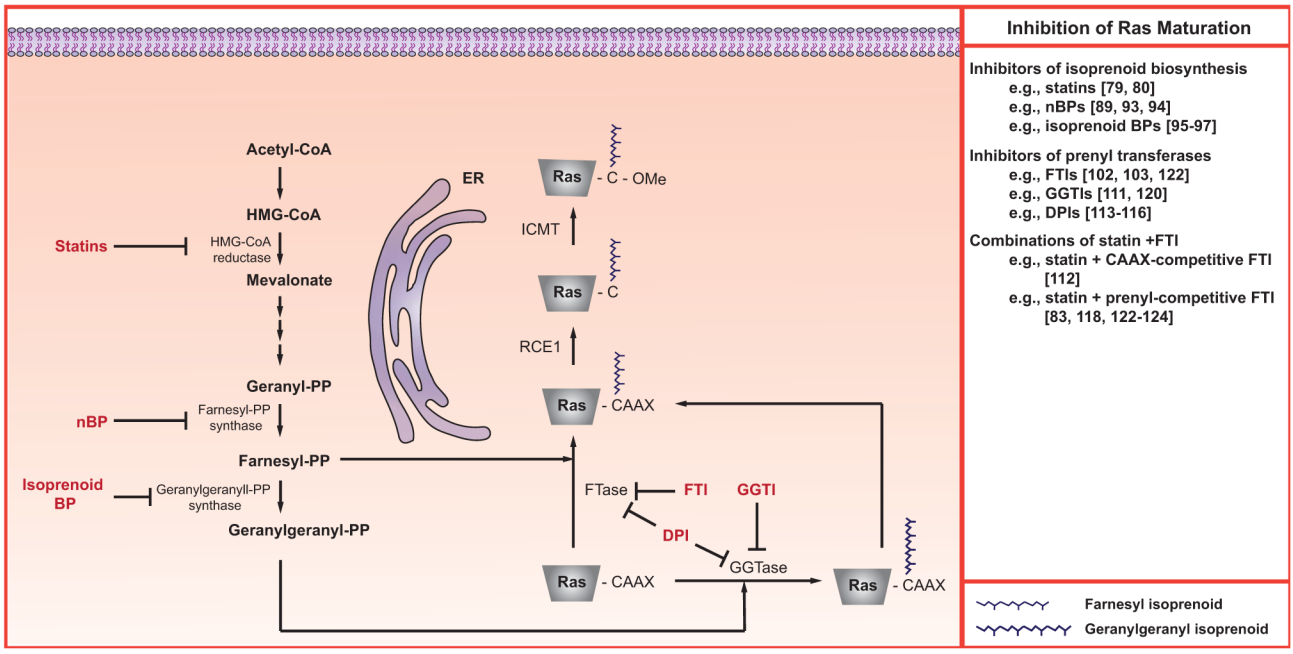

Fig. (2). Inhibition of Ras maturation

Newly-synthesized Ras polypeptides undergo complex, multi-step maturation pathways. Many of the enzymes shown have been considered as potential therapeutic targets. In addition to those detailed in this figure, both Rce1 and Icmt have also been investigated [see 5 for review].

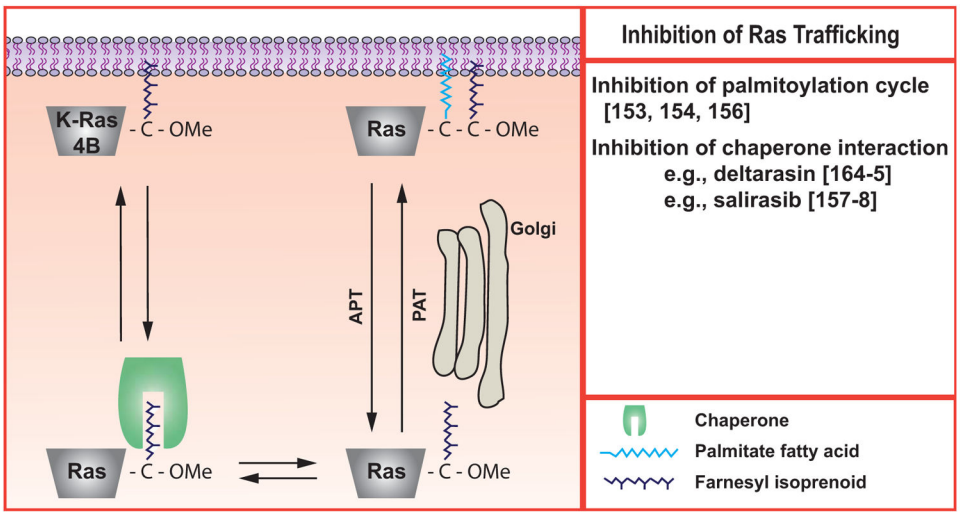

Fig. (3). Inhibition of Ras trafficking

The prenylated Ras proteins require additional steps to achieve the membrane localization that is required for their activity. H-Ras, K-Ras4A, and N-Ras are reversibly palmitoylated. K-Ras4B requires interactions with chaperone proteins such as galectins or PDEδ. This figure is adapted and re-drawn from [156].