Targeting RAS Membrane Association: Back to the Future for Anti-RAS Drug Discovery?

Adrienne D. Cox1, Channing J. Der1, and Mark R. Philips2

Abstract

RAS proteins require membrane association for their biologic activity, making this association a logical target for anti-RAS therapeutics. Lipid modification of RAS proteins by a farnesyl isoprenoid is an obligate step in that association, and is an enzymatic process. Accordingly, farnesyltransferase inhibitors (FTI) were developed as potential anti-RAS drugs. The lack of efficacy of FTIs as anticancer drugs was widely seen as indicating that blocking RAS membrane association was a flawed approach to cancer treatment. However, a deeper understanding of RAS modification and trafficking has revealed that this was an erroneous conclusion. In the presence of FTIs, KRAS and NRAS, which are the RAS isoforms most frequently mutated in cancer, become substrates for alternative modification, can still associate with membranes, and can still function. Thus, FTIs failed not because blocking RAS membrane association is an ineffective approach, but because FTIs failed to accomplish that task. Recent findings regarding RAS isofrom trafficking and the regulation of RAS subcellular localization have rekindled interest in efforts to target these processes. In particular, improved understanding of the palmitoylation/depalmitoylation cycle that regulates RAS interaction with the plasma membrane, endomembranes, and cytosol, and of the potential importance of RAS chaperones, have led to new approaches. Efforts to validate and target other enzymatically regulated posttranslational modifications are also ongoing. In this review, we revisit lessons learned, describe the current state of the art, and highlight challenging but promising directions to achieve the goal of disrupting RAS membrane association and subcellular localization for anti-RAS drug development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Learning Objectives

Upon completion of this activity, the participant should have a better understanding of the strategies currently under investigation to improve treatment of RAS-driven cancers, and of the biologic rationale underlying novel therapeutic strategies that disrupt RAS membrane association.

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Introduction

The three RAS genes (HRAS, NRAS, KRAS) are the most commonly mutated oncogenes in human cancers (1, 2). The role of oncogenic RAS proteins as key drivers in both common and uncommon cancers has led to intensive efforts over more than three decades to develop therapeutics that target RAS, encompassing both direct and indirect approaches. Oncogenically mutated RAS proteins fail to cycle *off* from the active

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GTP-bound state to the resting GDP-bound state, and thereby accumulate in the "on" configuration. Early direct approaches sought to attack this impaired molecular switch. Attempts to identify antagonists of GTP binding or to identify drug-like mimics of the negative regulatory GAP proteins have been unsuccessful, although new strategies of stabilizing conformational states may yet bear fruit, as discussed elsewhere in this CCR Focus section (3). More recent efforts to target specific RAS mutations (e.g., KRAS G12C), to interfere with RAS binding to its activator SOS1, and to block association with effectors such as RAF1 have been reviewed recently (1, 4). The consensus at present is that the most fruitful direction for anti-RAS therapeutics in the near future is indirect targeting of RAS signaling via inhibiting its downstream effectors, particularly the RAF–MEK–ERK and PI3K–AKT–MTOR kinase cascades that have been shown to be critical for RAS driver functions in specific cancers. These efforts are discussed elsewhere (1, 4, 5). Other approaches, such as attempts to identify additional targets for coinhibition with RAS, through synthetic lethality screens or metabolic dependencies, are also discussed elsewhere in this CCR Focus section (6, 7). Here, our focus is on direct targeting of RAS by interfering with its membrane association and trafficking. We argue that this approach, while challenging, remains both logical and potentially tractable, given information that has emerged over the past few years. Because the association of RAS proteins with membranes is absolutely required for their function, targeting this requirement can be viewed as the functional equivalent not of turning off the defective switch that is oncogenic RAS, but of removing it and thereby destroying the circuit.

CAAX Processing and RAS Membrane Association

The critical need for RAS protein association with cellular membranes has been appreciated for decades (8, 9). RAS association with the plasma membrane and with other membrane compartments upon which signaling occurs (10, 11) is promoted by a well-described series of posttranslational modifications at RAS C-terminal CAAX motifs (Fig. 1), where C = cysteine,
isoprenoid lipid to the cysteine of the CAAX motif. This provides them sufficient affinity for the endoplasmic reticulum (ER), where they are further modified by RAS-converting CAAX endopeptidase 1 (RCE1)-catalyzed proteolytic removal of the AAX residues, and by reversible isoprenylcysteine carboxymethyltransferase (ICMT)-catalyzed carboxylmethylation of the now terminal farnesylated cysteine residue. By preventing the first and obligate step, FTIs prevent all three of these modifications. In the presence of FTIs, KRAS and NRAS are alternatively prenylated by geranylgeranyltransferase I (GGTase I), which attaches a C20 geranylgeranyl isoprenoid that allows the same subsequent processing steps. RAS trafficking to the inner leaflet of the plasma membrane (PM) requires a second membrane-targeting element that dictates the pathway it will take to the plasma membrane. NRAS and HRAS have one or two cysteine residues, respectively, that undergo reversible acylation by a Golgi-resident protein acyltransferase (PAT) to promote their trafficking to the plasma membrane. Rapid deacylation by an acyl protein thioesterase (APT1/2) frees them up to be re-acylated and trafficked back to the plasma membrane. The nonpalmitoylated pool of APT in the cytosol is the active form, and in is in dynamic equilibrium with a palmitoylated pool on the Golgi. KRAS4B, which has no palmitoylatable cysteine but a stretch of 6 lysines (polybasic region) does not go to the Golgi but trafficks the more directly to the plasma membrane, where it binds by virtue of its electrostatic charge. KRAS4A, which has a hybrid motif of a palmitoylated cysteine and a bifurcated polybasic region, undergoes an intermediate form of trafficking. PDE6A recognizes the farnesyl isoprenoid and solubilizes nonpalmitoylated RAS proteins from any compartment, thereby promoting their availability for restoration to the plasma membrane. Deltarasin blocks this interaction. Not pictured: other chaperone proteins that guide the lipidated RAS proteins between and within membrane regions. Each enzyme depicted has been a target for drug discovery.

Targeting CAAX Prenylation: FTase and GGTase, Statins

RAS farnesylation by FTase is the first, irreversible, and rate-limiting step of CAAX processing. As it was quickly determined to be both an obligate modification for oncogenic RAS biologic activity and a process governed by an enzyme that recognized a simple short tetrapeptide CAAX motif, this step was rapidly exploited for inhibition. Both rational drug design and library screening were employed in numerous intensive and successful efforts to identify FTase inhibitors (FTI; refs. 16, 17). Two of these, lonafarnib and tipifarnib, progressed to advanced clinical trials but failed to show efficacy against KRAS-driven cancers (16, 17). The failure of FTIs, once anticipated to be magic bullets for RAS-driven cancers, to serve as broadly effective anti-RAS drugs led to a widespread misperception that even RAS itself is not a good target.

**Figure 2.**
RAS trafficking pathway. Nascent RAS proteins leaving the polysome are rapidly modified by farnesyltransferase (FTase), which attaches a C15 farnesyl isoprenoid lipid to the cysteine of the CAAX motif. This provides them sufficient affinity for the endoplasmic reticulum (ER), where they are further modified by RAS-converting CAAX endopeptidase 1 (RCE1)-catalyzed proteolytic removal of the AAX residues, and by reversible isoprenylcysteine carboxymethyltransferase (ICMT)-catalyzed carboxylmethylation of the now terminal farnesylated cysteine residue. By preventing the first and obligate step, FTIs prevent all three of these modifications. In the presence of FTIs, KRAS and NRAS are alternatively prenylated by geranylgeranyltransferase I (GGTase I), which attaches a C20 geranylgeranyl isoprenoid that allows the same subsequent processing steps. RAS trafficking to the inner leaflet of the plasma membrane (PM) requires a second membrane-targeting element that dictates the pathway it will take to the plasma membrane. NRAS and HRAS have one or two cysteine residues, respectively, that undergo reversible acylation by a Golgi-resident protein acyltransferase (PAT) to promote their trafficking to the plasma membrane. Rapid deacylation by an acyl protein thioesterase (APT1/2) frees them up to be re-acylated and trafficked back to the plasma membrane. The nonpalmitoylated pool of APT in the cytosol is the active form, and in is in dynamic equilibrium with a palmitoylated pool on the Golgi. KRAS4B, which has no palmitoylatable cysteine but a stretch of 6 lysines (polybasic region) does not go to the Golgi but trafficks the more directly to the plasma membrane, where it binds by virtue of its electrostatic charge. KRAS4A, which has a hybrid motif of a palmitoylated cysteine and a bifurcated polybasic region, undergoes an intermediate form of trafficking. PDE6A recognizes the farnesyl isoprenoid and solubilizes nonpalmitoylated RAS proteins from any compartment, thereby promoting their availability for restoration to the plasma membrane; deltarsarin blocks this interaction. Not pictured: other chaperone proteins that guide the lipidated RAS proteins between and within membrane regions. Each enzyme depicted has been a target for drug discovery.
However, it is critical to recognize that, in the presence of FTIs, NRAS and KRAS, but not HRAS, become substrates for geranylgeranyltransferase 1 (GGTase 1) through a process known as alternative prenylation (18, 19). This phenomenon was revealed only when FTIs became available. Because geranylgeranylated RAS proteins still associate with membranes and are still biologically active, FTIs were ineffective despite hitting their FTase target. Thus, FTIs failed as anti-RAS drugs not because blocking RAS association with membranes is a flawed approach, but rather because FTIs failed to achieve this goal. Accordingly, we and others have continued to pursue the RAS modification and trafficking pathways as logical targets for potential therapeutics. One potential solution to the problem of alternative prenylation might be dual inhibition of FTase and GGTase 1 (20), either by combining individual inhibitors of each enzyme, or by dual specificity inhibitors. Such inhibitors have been developed and some have reached the clinic (16,17), but have been limited by toxicity (21–23). There are still hopes that a therapeutic window can be found, possibly by targeting their delivery to RAS-driven cancer cells (17), where oncogenic RAS rendered cytosolic by these agents could sequester effectors and act as dominant negatives (24,25).

Because farnesyl pyrophosphate (FPP) is both an intermediate in the cholesterol biosynthetic pathway and is also used for modification of CAAX proteins, it has long been speculated that statins may limit cancer by inhibiting prenylation of RAS or related small GTPases. However, due to the differential Km for squalene synthase versus FTase, cholesterol synthesis is 1,000-fold more easily inhibited by loss of FPP than is FTase-mediated modification of RAS (26). Consistent with this, only suprapharmacologic levels of statins block protein prenylation in cell culture (27) and cause mislocalization of RAS (28), whereas therapeutic levels do not (28). Moreover, the effects of statins on cell growth are RAS-independent (27); nor is there evidence for these effects on RAS in animals administered pharmacologically relevant doses. Thus, statins show no promise for use as anti-RAS drugs.

**Targeting Postprenylation CAAX Processing: RCE1 and ICMT**

Although it is clear that the postprenylation CAAX-processing enzymes also contribute to RAS membrane association, making them potentially attractive targets for drug development, there are numerous challenges to overcome before inhibition of either RCE1 or ICMT can be translated to the clinic. These include uncertainty regarding the requirements for RCE1 and ICMT in oncogenic RAS functions, perplexing results from genetic validation studies, lack of understanding of the mechanism of RCE1 CAAX protease activity, and difficulties in developing inhibitors of these enzymes that can achieve sufficient potency and selectivity to become clinically efficacious drugs. Nevertheless, resolving these issues is likely to reveal important facets of RAS biology that may in turn reveal additional targets, warranting some discussion here.

RCE1 generates the substrate for ICMT: a prenylcysteine with an α-carboxyl group. Thus, ICMT can act only after RCE1. The sequential nature of these modifications implies that RCE1 deficiency should produce cell biologic consequences at least as strong as ICMT deficiency, if one assumes that the effects of partial processing are similar for each substrate. At present, due to the lack of drug-like potent and selective inhibitors, this can be tested genetically but not pharmacologically. These studies reveal significant context dependence of deficiencies in these enzymes. Contrary to initial suppositions, Icmt-null mice die earlier in gestation than do Rce1-null mice (29,30), and Icmt deficiency completely blocked the transformed growth of RAS-transformed rodent fibroblasts, whereas Rce1 deficiency had modest effects (31). Furthermore, the consequences of genetic loss of Rce1 and Icmt can be both opposing and context dependent. In a mouse model, the same myeloproliferative disease driven by oncogenic Kras G12D that was ameliorated by Icmt deficiency (32) was enhanced by Rce1 deficiency (33). However, Icmt deficiency accelerated the disease in a mouse model of pancreatic cancer driven by Kras G12D (34), due to inhibition of signaling from Notch-1, which acts as a tumor suppressor in this model (35). Importantly, Icmt deficiency ameliorated Kras-driven disease in other mouse tumor models (M.G. Dalin and M.O. Berg; unpublished results), supporting the context dependency of ICMT impairment. These results suggest that a better understanding of the biology of CAAX processing will be critical to further validation of RCE1 and ICMT as potential drug targets, particularly with respect to the indications and populations in which they may be best applied. The complexity inherent in the context-dependent roles of RCE1 and ICMT is likely explained by the myriad substrates of these enzymes other than RAS, many of which are signaling molecules.

Many drugs fail in development due to cardiac toxicity, which is a demonstrated consequence of Rce1 depletion in the heart (36). Similarly, targeted depletion of Rce1 in the retina resulted in rapid degeneration of specific photoreceptor cells (37). On the other hand, the cleavage of RHOA by the bacterial toxin and cysteine protease YopT requires RCE1 but not ICMT (38). Thus, even if they can be developed, RCE1 inhibitors may be more toxic overall than ICMT inhibitors. Nevertheless, attempts have been made to develop inhibitors of both RCE1 (39) and ICMT (40–42), although each will necessarily also affect alternate substrates in addition to RAS. Disappointingly, even the most potent and selective of the RCE1 inhibitors have recently been shown to lack mechanism-based activity (43). The antiproliferative effects of ICMT inhibitors on RAS-transformed cells may be more compelling (40); whether they can be converted into pharmacologic leads is currently unclear.

**RAS Trafficking: “Second Signals,” the Acylation/Deacylation Cycle, and RAS Chaperones**

RAS membrane association and trafficking are regulated in a complex manner that has yet to be fully unraveled (Fig. 2). This complexity has revealed both additional challenges and additional targets for drug discovery. First, in addition to modification of the CAAX sequence, RAS plasma membrane association requires a proximal ‘second signal’ that is either palmitoylation of one (NRAS) or two cysteines (HRAS) or a polybasic stretch of lysine residues (KRAS4B; refs. 44, 45) to confer additional hydrophobicity or an electrostatic interaction with the negatively charged headgroups of the phospholipids at the inner leaflet of the plasma membrane, respectively (Fig. 1). KRAS4A is unique among the four RAS proteins in possessing a dual membrane targeting motif that consists of both a palmitoylated cysteine and two short polybasic regions flanking that acylated cysteine
Targeting RAS Trafficking by Disrupting Depalmitoylation: Protein Acyl (Palmitoyl) Transferases

Aside from CAAX-signaled modifications, enzymatic targets in the RAS processing pathway include those that regulate the palmitoylation/depalmitoylation cycle. RAS, the predominant RAS gene mutated in human cancers, is expressed in two splice variants. The nonpalmitoylated KRAS4B has been generally accepted to be the major driver of cancer. However, recent work implicating the palmitoylated splice variant KRAS4A in colorectal adenocarcinoma (46) may force a re-evaluation of the notion that palmitoylation is not a good target in RAS-driven cancers. Moreover, palmitoylation inhibitors may help to limit the activity of oncogenic NRAS (64, 65), a key driver of melanoma and hematopoietic malignancies. As inhibitors of palmitoylation and depalmitoylation are developed, it may also be important to better understand the roles of palmitoylated wild-type RAS isoforms in supporting or impairing oncogetic RAS-driven cancers (1). Non-specific inhibitors of protein palmitoylation such as 2-bromo-palmitate (66) have been useful as tool compounds, but are unlikely to be developed into drugs given the vast number of palmitoylated RAS proteins in the human genome (67). The recent identification of 23 DHHC (aspartic acid-histidine-histidine-cysteine tetrapeptide motif) proteins in the mammalian repertoire of protein acetyltransferases (PAT) supports the feasibility of developing palmitoylation inhibitors that are specific for a subset of substrates such as RAS proteins (68). Consensus motifs to link specific palmitoylated substrates with their respective PATs have not yet been determined, but identification of the DHHC9/GPC16 complex as the PAT that modifies NRAS and HRAS (47) suggests the possibility of targeting this process selectively. Although progress in this direction has been limited to date (66, 68, 69), the recent advent of metabolic labeling with the bioorthogonal fatty acid 17-octadecynoic acid (17-ODYA) followed by click chemistry-mediated retrieval of labeled substrates promises to simplify transferase assays that can be applied to compound screens. In addition, because sequences around the palmitoylation sites stabilize membrane association and signaling of palmitoylated RAS proteins without affecting palmitoylation status itself (70, 71), these may also need to be taken into account for successful PAT inhibitor development.

Targeting RAS Trafficking by Disrupting Depalmitoylation: APT1/LYPLA1 Acyl Protein Thioesterases

There has been more progress in the area of inhibiting depalmitoylation, although translating the present tool compounds to drugs is also likely to be challenging. Counterintuitive although it may seem at first glance, the rationale for inhibiting depalmitoylation of palmitoylated RAS proteins is that depalmitoylation on all cellular membranes seems to be required for dynamic cycling of NRAS and HRAS among their membrane locations (72). This process is facilitated by acyl protein thioesterase 1 (APT1, also known as lysophospholipase 1, LYPLA1) and possibly also APT2, which are themselves reversibly S-acylated (73). The existence of APT in both palmitoylated Golgi-bound and nonpalmitoylated cytosolic pools (74) due to autodepalmitoylating activity of APTs (74) may explain how cytosolic (nonpalmitoylated) APT can promote cycling of palmitoylatable isoforms of RAS proteins to and from membranes, ultimately resulting in enrichment of the palmitoylated forms at the plasma membrane and Golgi (72, 74). These features provide the rationale for development of APT1/2 inhibitors as potential anti-RAS agents. The ability of a series of β-lactone-based inhibitors such as palmositarn B and M (derived from the over-the-counter weight loss drug tetrahydrolipstatin; refs. 75, 76) to mislocalize HRAS and NRAS proteins to internal membranes (75, 76) and to inhibit the proliferation of myeloid progenitor cells expressing oncoenic NRAS in treated mice (77) suggests that such inhibitors have the potential to disrupt oncoenic NRAS function, although questions remain regarding kinetics and target specificity (78). Additional tool compounds such as boron-based APT1/2 inhibitors have been identified (79). Other recent screens have identified LYPLA1/- and LYPLA2-selective inhibitors based on a triazole urea scaffold (80). Interestingly, APTs are not the only regulators of RAS depalmitoylation; for example, HRAS becomes depalmitoylated following the peptidyl-prolyl isomerase activity of FKBP12 on a proline near the palmitoylated cysteines (81). Understanding and optimizing the possible effects of such inhibitors may be complicated by the differential dynamics of wild-type and oncoenic RAS palmitoylation (82), and by the numerous non-RAS substrates of APT1/2/LYPLA1/2 that also undergo dynamic palmitoylation, including proteins as varied as heterotrimeric G protein alpha subunits, MAGUK scaffolds, Src family tyrosine kinases, nucleopory, and BK ion channels (69). Nevertheless, the advent of new labeling technologies and new probes are making it possible to better study dynamic palmitoylation (83), which in turn is expected to reveal novel paths toward improved inhibitors of this key modification.

Targeting RAS Trafficking by Disrupting Other Modifications: PKC-Mediated Phosphorylation

In addition to CAAX processing and palmitoylation, other posttranslational modifications of RAS are potentially targetable (1, 84). Of these, phosphorylation of KRAS4B on serine 181, a process that is mutually exclusive with calmodulin binding (85),
can alter subcellular localization dramatically, displacing the modified GTPase from the plasma membrane (50, 86), and converting KRAS4B from a growth-promoting to a growth-suppressing protein (87). Consistent with this, a PKC agonist, bryostatin, slowed the growth of mouse tumors driven by oncogenic KRAS4B but not those driven by a phosphorylation-deficient KRAS4B mutant (50). It is unclear why, in another study, rodent fibroblasts transformed with phosphorylation-deficient KRAS4B/D12V, S181A failed to produce tumors in nude mice (88), leading these investigators to conclude that phosphorylation of KRAS4B is required for oncogenesis rather than leading to growth suppression. We have found that crossing p48-Cre mice with animals with a phosphorylation-deficient LSL-Kras4B-G12D/S181A double knock-in allele produced pancreatic tumors with equal frequency as crossing to mice with a phosphorylatable LSL-Kras4B-G12D/S181S allele (unpublished results), arguing strongly against a requirement for KRAS4B phosphorylation. Importantly, a recent study (89) showed that numerous cancer-associated mutations of PKC isoforms are loss-of-function mutations. This finding suggests that these PKCs act as tumor suppressors, consistent with the effect of phosphorylating KRAS4B on S181. Regardless of the explanation, although bryostatin and analogues have been under preclinical and clinical investigation as anticancer treatments, are reasonably well tolerated and have antitumor activity (90), enthusiasm for this approach to anti-KRAS therapy is diminished by the low probability of finding a drug that stimulates KRAS4B phosphorylation without affecting PKC-mediated activation of other signaling molecules that promote tumor growth and/or lead to toxicities.

Targeting RAS Trafficking by Disrupting RAS–Chaperone Interactions

In addition to blocking enzymatic activities regulating RAS membrane interactions, several distinct approaches have been taken to disrupting farnesylated RAS binding to chaperones, largely with the intent to block oncogenic KRAS4B specifically. Inhibitors of the Ras–PDE6b interaction have recently been identified. Deltarasin, at low micromolar levels, impaired the accumulation of KRAS4B on the plasma membrane and slowed the growth in vitro and in vivo of a tumor cell line harboring mutant KRAS (62, 91). However, the degree of dependence of KRAS on PDE6b is not yet clear. For example, PDE6b knockout mice are viable and fertile (92), whereas knockout of Kras in mice is embryonic lethal (93), indicating that KRAS can still function in the absence of PDE6b. On the other hand, the crystal structure of PDE6b indicates little to no specificity for one farnesylated protein over another (63), which may be reflected in the discordant observations of RAS interactions mentioned above. It may also be that, similarly to effective multikinase inhibitors that were once disparaged as “dirty” drugs, inhibition of multiple farnesylated proteins contributes to the salutary effects of deltarasin. Future translation of current PDE6b inhibitors to clinical leads will likely require a better understanding of its chaperone specificity (94) and trafficking patterns.

Salirasib, also known as farnesylthiosalicylic acid (FTS), resembles the S-farnesylated cysteine on Ras (95, 96) and is proposed to compete with it for binding to chaperones such as galectins (52, 56, 97). A small clinical trial showed that salirasib was well tolerated in pancreatic cancer patients (98), yet definitive answers as to whether it effectively perturbed KRAS function in patients, and can provide clinical benefit, remain to be determined. Like the other inhibitors of RAS membrane association, salirasib is not specific for RAS, but also inhibits other farnesylated proteins, including RHEB and MTOR (1). Larger trials are merited, but await a better understanding of salirasib mechanism of action and elucidation of tractable biomarkers.

Meanwhile, galectins are also known to modulate RAS nanoclustering and localization within defined membrane microdomains (53–56, 99), that in turn are regulated by phosphatidyserine (99). Thus, perhaps it was not surprising that an unbiased high-content screen to observe mislocalization of KRAS4B from plasma membrane to endomembranes revealed that staurosporine and analogues blocked endosomal recycling of phosphatidyserine and displaced KRAS4B from the plasma membrane to endosomes where it was degraded (100). Interestingly, fendiline, an L-type calcium channel blocker, was also identified as a compound that induced KRAS4B mislocalization (101); however, this activity was channel independent. The third class of compound identified in this visual screen was metformin, that also displaced KRAS4B from the plasma membrane (102). These findings are very exciting because they suggest that unbiased screens have the potential to uncover existing drugs that can be repurposed to block KRAS4B membrane association. To further identify new regulators of KRAS4B membrane association, the Philips lab has designed a genome-wide siRNA screen employing a dual luciferase assay that quantitatively reports displacement of KRAS4B from cellular membranes (unpublished data). We expect that additional roles for still other proteins in membrane targeting and/or stabilization have yet to be revealed. As this new information adds to the surge in understanding how nascent RAS proteins are delivered to the plasma membrane, we expect the RAS trafficking pathway to continue to provide a target-rich environment for drug discovery.

Authors’ Contributions

Conception and design: A.D. Cox, C.J. Der, M.R. Philips
Writing, review, and/or revision of the manuscript: A.D. Cox, C.J. Der, M.R. Philips

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