

Polyisoprenylated Cysteinyl Amide Inhibitors: A Novel Approach to Controlling Cancers with Hyperactive Growth Signaling



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Abstract: Aberrant activation of monomeric G-protein signaling pathways drives some of the most aggressive cancers. Suppressing these hyperactivities has been the focus of efforts to obtain targeted therapies. Polyisoprenylated methylated protein methyl esterase (PMPMEase) is overexpressed in various cancers. Its inhibition induces the death of cancer cells that harbor the constitutively active K-Ras proteins. Furthermore, the viability of cancer cells driven by factors upstream of K-Ras, such as overexpressed growth factors and their receptors or the mutationally-activated receptors, is also susceptible to PMPMEase inhibition. Polyisoprenylated cysteinyl amide inhibitors (PCAI) were thus designed to target cancers with hyperactive signaling pathways involving the G-proteins. The PCAI were, however, poor inhibitors of PMPMEase, with K_i values ranging from 3.7 to 20 μM . On the other hand, they inhibited cell viability, proliferation, colony formation, induced apoptosis in cells with mutant K-Ras and inhibited cell migration and invasion with EC_{50} values of 1 to 3 μM . HUVEC tube formation was inhibited at submicromolar concentrations through their disruption of actin filament organization. At the molecular level, the PCAI at 2 to 5 μM depleted monomeric G-proteins such as K-Ras, RhoA, Cdc42 and Rac1. The PCAI also deplete vinculin and fascin that are involved in actin organization and function while disrupting vinculin punctates in the process. These demonstrate a polyisoprenylation-dependent mechanism that explains the observed PCAI inhibition of the proliferative, invasive and angiogenic processes that promote both tumor growth and metastasis.

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1. INTRODUCTION

An estimated 2% of eukaryotic proteins are polyisoprenylated [1]. Polyisoprenylation, also known as prenylation or isoprenylation, involves the biochemical modification of the proteins with *trans*, *trans*-farnesyl, or all *trans*-geranylgeranyl groups [2, 3]. Three enzyme-catalyzed steps are involved in the biotransformation process for the subtype of proteins with the CAAX carboxyl-terminal signal sequence whereby C is cysteine, A represents an aliphatic amino acid and X could be M, S, Q, A, C, L or E signaling for the polyisoprenylation [4, 5]. When X is M, S, Q, A or C, the proteins undergo farnesylation while L or E results in geranylgeranylation in reactions catalyzed by farnesyl

transferase and geranylgeranyl transferase I, respectively [6-8]. The requirement for polyisoprenylation of Ras and related G-proteins for malignant transformation spurred scientists to develop polyisoprenylation inhibitors for anticancer therapy [9]. The use of Farnesylation Inhibitors (FIs) to block the secondary modifications resulted in the alternative geranylgeranylation of K- and N-Ras [10], culminating in the loss of the potential anticancer benefits [11]. Geranylgeranyl transferase I inhibitors have, therefore been introduced into the antitumor drug development strategy [12, 13].

Following polyisoprenylation, the AAX tripeptide of the signal sequence is cleaved by Ras Converting Enzyme 1 (RCE1), thereby exposing the carboxyl terminal cysteine. The cysteine carboxylate undergoes methylation catalyzed by polyisoprenylated protein methyltransferase (PPMTase, also known as isoprenylcysteine methyl transferase or ICMT) [8]. A demethylating enzyme (PMPMEase) has been found to hydro-

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lyze this methyl ester [14, 15]. The charge on the terminal polyisoprenylated cysteine may be pivotal in regulating the activities of these proteins. Moreover, electrostatic forces exerted on the carboxylate likely affect the spatial orientation of the polyisoprenyl group as the proteins undergo methylation/demethylation reactions. As polyisoprenyl-binding pockets have been revealed in some proteins [16-18], these secondary modifications are essential for the protein-protein interactions and biological activities of the proteins involved. Therefore, molecules that inhibit the polyisoprenylation pathway enzymes have long been sought to modify the proteins' functional interactions.

This then brings into focus the role of the understudied demethylating enzyme, polyisoprenylated methylated protein methyl esterase (PMPMEase) that has been a major focus of the studies in our laboratory. PMPMEase is overexpressed in cancers including pancreatic [19], prostate [20], colorectal [21] and lung [22]. Cell lines from these cancer types are all susceptible to PMPMEase inhibition with polyisoprenylated small-molecule irreversible inhibitors [20, 22, 23]. The Polyisoprenylated Cysteiny Amide Inhibitors (PCAI)s were designed to target and inhibit this enzyme in order to mitigate the hyperactive growth signaling in various cancers [24]. This review focuses mainly on PMPMEase, the molecules that we have designed in order to target it for inhibition, and observed cancer biological and molecular changes they induce in cancer cell lines.

2. POLYISOPRENYLATED METHYLATED PROTEIN METHYL ESTERASE

2.1. Role of PMPMEase in the Polyisoprenylation Pathway

The final step of the polyisoprenylation pathway is an S-adenosyl-L-methionine (SAM)-dependent methylation reaction catalyzed by PPMTase [25-30]. The methylation converts the negatively charged carboxylate into the uncharged methyl ester (Fig. 1). It has been speculated that this net charge difference between the methylated and demethylated forms of polyisoprenylated proteins may have an impact on their functional conformations [31]. That this may be the case has been substantiated by reports of polyisoprenyl binding sites on some proteins [17, 18]. Loss of PPMTase has serious physiological consequences. For example, PPMTase knockouts in mice are lethal by mid-gestation [32], while PPMTase inactivation leads to mislocalization of K-Ras from the cell membrane [33].

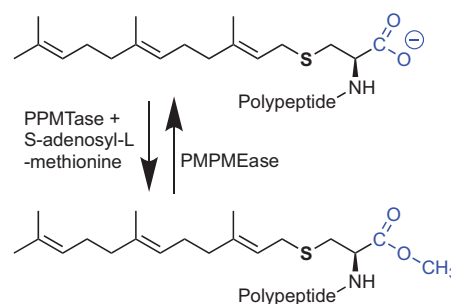


Fig. (1). Polyisoprenylation-dependent methylation and demethylation reactions. SAM-dependent methylation removes a negative charge in the vicinity of the polyisoprenyl moiety that is important for protein-protein interactions. Protein conformational changes resulting from methylation/demethylation-induced electrostatic interactions could alter the spatial orientations of the polyisoprenyl moiety relative to the polypeptide portions of the proteins, making them sterically unavailable for the protein-protein interactions.

The significance of the loss of PPMTase activity in animal physiology underscores the importance of a carefully regulated equilibrium between the methylated and demethylated forms of polyisoprenylated proteins. The methylation step is the only reversible reaction of the pathway as hydrolysis of the methyl esters regenerates the demethylated proteins. As such, this has been proposed to control polyisoprenylated protein function [29]. While this possibility has been recognized for a long time, interest in PMPMEase, the enzyme that counterbalances the effects of PPMTase has been very limited. Judging from the detrimental effects of PPMTase knockouts on mouse embryonic development [32], a pertinent question is to know whether a hyperactive PMPMEase that overwhelms PPMTase activity may be similarly impactful physiologically. Also of interest is how low PMPMEase activities may impact cell growth and animal physiology.

To begin addressing some of these questions, our earlier work sought to identify and characterize PMPMEase. The original interest in PMPMEase stemmed from the observation that excess SAM caused the carboxymethylation of proteins similar to the heterotrimeric G-protein γ -subunits in rat brains. This resulted in Parkinson's disease-like phenomena that were blocked by prior treatment with farnesylated compounds [34-36]. We reasoned that if excess protein methylation could cause these effects, then too little demethylation caused by low PMPMEase activities might have similar disease consequences. This led to the initial work of characterizing PMPMEase that revealed it as a serine hydrolase based on its susceptibility to irreversible serine hydrolase inhibitors [15]. This characterization as a carboxylesterase was later confirmed when it was purified from the porcine liver and

the trypsin digest sequenced by tandem mass spectrometry [15] and found to be *Sus scrofa* carboxylesterase 1.

2.2. The Nature of PMPMEase

To begin to understand the role of PMPMEase in cellular functions, specific inhibitors were needed for this purpose. Using a *trans, trans*-farnesylated substrate to screen chromatographic fractions for PMPMEase activity, a range of substrates was developed to probe the active site of the enzyme to aid in the design of high-affinity inhibitors. These studies revealed that PMPMEase has a 320-fold higher affinity for *trans, trans*-farnesylated and all *trans*-geranylgeranylated substrates compared to substrates with shorter alkyl groups [37]. This was consistent with numerous x-ray crystallographic studies on human Carboxylesterase 1 (hCE1), revealing the hydrophobic nature and flexibility of the active site [38-41], possibly for binding the polyisoprenyl group and accommodating the varied polypeptide portions of endogenous substrates, respectively. These crystallographic data have contributed immensely in the docking analyses of substrates and inhibitors-binding interactions studies [23, 24, 42].

3. DESIGNING SPECIFIC INHIBITORS OF PMPMEASE

3.1. Sulfonyl Fluorides as Mechanism-based Inactivators

Significant insights into the design of specific high-affinity inhibitors of PMPMEase came from the substrate kinetics studies that demonstrated the requirement for the polyisoprenyl moiety for both selectivity and high-affinity interactions [37, 42]. The polyisoprenyl moiety has thus been an essential component of all the compounds that have been designed to target PMPMEase for inhibition. Using the polyisoprenyl group as the principal component of inhibitor design that defines selectivity for PMPMEase, other bioisosteric functional groups were then used to complete the inhibitor design. Given that PMPMEase is inhibited irreversibly by phenylmethylsulfonyl fluoride (PMSF) and organophosphorus compounds, the initial group of inhibitors incorporated the sulfonyl fluoride moiety as a reactive “warhead”. Since the inhibition mechanism relies on the inhibitors acting as pseudo-substrates, the “warheads” were precisely spaced from the polyisoprenyl moiety as the susceptible carboxyl esters of the substrates. The high-affinity interactions of the polyisoprene with the hydrophobic pockets of the binding site implies that the “warheads” were in close proximity to the catalytic residues for mechanism-based inactiva-

tion to occur (Fig. 2). This approach led to the sulfonyl fluoride irreversible inhibitors of PMPMEase [23]. The most effective of the sulfonyl fluorides, L-28, incorporated the sulfonyl fluoride moiety as a bioisosteric replacement of the carbonyl moiety of the substrates (Fig. 2) [23, 43].

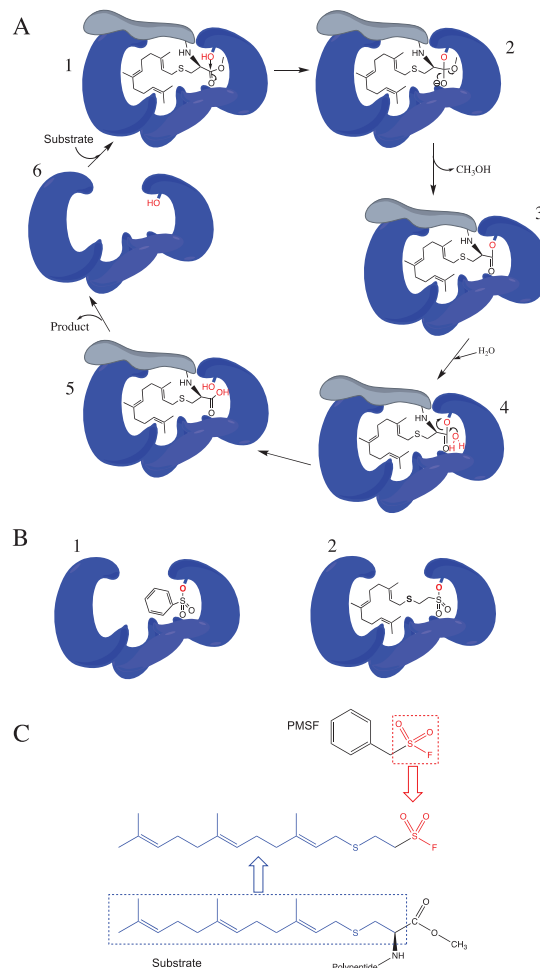


Fig. (2). PMPMEase enzymatic mechanism and the design of the irreversible polyisoprenylated sulfonyl fluoride inhibitor of PMPMEase. (A) Upon substrate binding (step 1), PMPMEase employs the catalytic serine residue to attack the carbonyl carbon of the susceptible ester bond (step 2), resulting in a transient acylated enzyme (step 3). This transition state is rapidly reversed by a water molecule (step 4), resulting in deacylation (step 5) and recovery of the enzyme (step 6). (B) Water is not a sufficiently strong nucleophile to displace the sulfonated enzyme. PMSF is thus an irreversible inhibitor of PMPMEase (1), a notion that was used in the design of the polyisoprenylated sulfonyl inhibitor, L-28 (2) according to the schematic (C). The polyisoprenyl moiety confers affinity towards the enzyme by the substrates and inhibitors. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

3.2. Polyisoprenylated Cysteinyl Amide Inhibitors (PCAI)s

Lack of aqueous solubility and moisture sensitivity are two important drawbacks that we encountered in the use of sulfonyl fluorides to study the role of PMPMEase in cells and animals. The lack of polar/ionizable groups limited the solubility in aqueous media while the fluoride ion is easily displaced by water resulting in a sulfonic acid product that lacks the ability to react with the active site catalytic serine hydroxyl group. The shortcomings led us to design the PCAIs as potential reversible PMPMEase inhibitors. An amide bond was used as a more stable bioisostere of the ester group in the substrates [24]. Given the essential nature of the polyisoprenyl moiety for high-affinity interactions and selectivity towards the enzyme, it also constituted an essential element of the PCAIs despite its hydrophobic nature. This requirement for the polyisoprenyl moiety for efficient metabolism by PMPMEase is shared with PPMTase [28]. In order to mitigate the hydrophobicity imparted by the polyisoprenyl and the cycloalkyl groups (denoted respectively by “A” and “D” in Fig. (3)), a tertiary amine moiety was incorporated as an ionizable appendage to improve the aqueous solubility of the PCAIs. It was envisaged that these

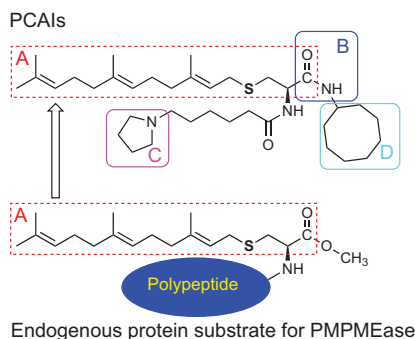


Fig. (3). Enzyme-substrate kinetics-based design of the PCAIs. The portion of the substrate, including the polyisoprenyl moiety (A) was maintained in the design while the ester functional group was replaced with the bioisosteric amide bond (B). The potential hydrophobicity of the PCAIs was mitigated by the inclusion of the ionizable side chain amines (C) that renders them aqueous-soluble making it possible for the non-pharmacophore A-B-D to be left intact. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

appendage groups are unlikely to sterically hinder the interactions of the PCAIs either with PMPMEase or other protein targets since it replaces the bulkier polypeptide portions of the endogenous protein substrates. When tested for their inhibition of PMPMEase and cancer cell viabilities, the PCAIs were found to be better at inhibiting cell viability than the enzyme, there-

by suggesting the potential involvement of PCAIs targets other than PMPMEase in the cancer cells as discussed in section 3.5.

3.3. Polyisoprenylated Proteins in Receptor Signaling and Cancer

In order to understand the potential role of the PCAIs against cancers, it is imperative to examine (1) the relevance of polyisoprenylated proteins in signaling pathways, (2) cancer drivers that appear upstream of and including the polyisoprenylated proteins and (3) protein-protein interactions that depend on the polyisoprenyl moieties of the proteins. Polyisoprenylated proteins are very important signaling molecules in various signaling pathways. Receptor tyrosine kinases require these proteins for transmitting various signals from extracellular growth factors into the cell. For example, the epidermal growth factor receptors require Ras as a downstream signaling mediator for cell proliferation, survival and cell cycle progression. Vascular endothelial growth factor receptors require proteins such as RhoA, Cdc42 and Rac1 for regulating actin polymerization into filopodia, lamellipodia and stress fibers necessary for maintaining cell shape and movement [44]. Cell movement is central to metastasis, the most devastating aspect of cancer since it promotes cell migration and invasion as well as cell reorganization during angiogenesis [45]. The latter is not only required for providing adequate nutrient supplies to fast-growing tumors but provides conduits for tumor cells to escape to form secondary tumors at distant sites.

3.4. Polyisoprenylated Protein-associated Cancer Drivers

Aberrant levels of expression and/or mutations resulting in hyperactive proteins that promote cancers have been reported for extracellular growth factors, their receptors and the downstream signaling intermediates that include polyisoprenylated proteins. Summarized in Table 1 are the incidence rates of some of the aberrant polyisoprenylated proteins in various cancers. Overexpression results in hyperactivities and increased signaling for cancer-promoting effects. Even more devastating are mutations that abolish the GTPase activities of some of the monomeric G-proteins. K-Ras mutations, in particular, have been reported to occur in over 50 and 90% of colorectal and pancreatic cancers, respectively, as well as to lesser extents in other cancers (Table 1). For example, while the percentage of lung cancer patients with hyperactive G-proteins may be relatively small, the fact that lung cancer afflicts a lot more people each year compared to other cancers, implies that hyperactive G-proteins is still a major problem in lung cancer.

Table 1. Some prominent G-protein cancer drivers and their incidence rates in human cancers. K-Ras, RhoA, Rac1 and Cdc42 are depleted in cancer cells treated with PCAIs resulting in loss of cancer progression phenomena such as cell viability, cytoskeletal organization, cell shape, motility and invasion, consistent with loss of their biological functions [24, 46-51].

| Protein | Cancer type | Aberrations (type of alteration) | Incidence rates (%) | References |
|---------|----------------------------------|----------------------------------|---------------------|------------|
| K-Ras | Pancreatic cancer | Mutation | 90 | [52] |
| | Non-small cell lung cancer | Mutation | 30-35 | [52] |
| | Breast | Mutation | 5 | [53] |
| | Colorectal cancer | Mutation | 30-54 | [52, 54] |
| | Eleven most common cancers | Mutation | 14.3 | [55] |
| N-Ras | Human cutaneous melanoma | Mutation | 94 | [56] |
| | Acute myelogenous leukemia (AML) | Mutation | 59 | [57] |
| H-Ras | Bladder urothelial | Mutations | 57 | [58] |
| RhoA | Colon | Overexpression | 95 | [59] |
| | Lung | Overexpression | 95 | [59] |
| RAC1 | Breast | Overexpression | 70 | [59, 60] |
| | | Mutation | 50 | [61, 62] |
| | Lung | Overexpression | 50 | [63] |
| | Melanoma | Mutation | 5 | [56] |
| Rac2 | Melanoma | Mutation | 10 | [56] |
| Cdc42 | Breast | Overexpression | 95 | [59] |
| | Colorectal | Overexpression | 60 | [64] |
| | Melanoma | Mutation | 5 | [56] |

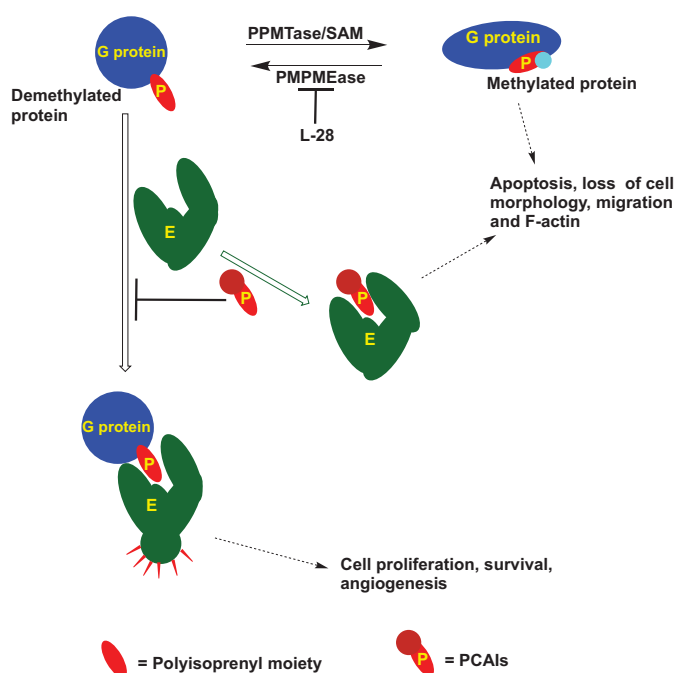


Fig. (4). Logical representation of the effects of treating cells with PMPMEase irreversible inhibitor, L-28 and PCAIs, and the possible interactions resulting in such effects. Some of the protein-protein interactions of the polyisoprenylated proteins are dependent on the polyisoprenyl moiety (P) [16]. The PCAIs possibly interfere with those interactions between the G-proteins and their effector (E) dependent on the polyisoprenyl cysteine, thereby disrupting the functional interactions and the subsequent events [46, 47, 50, 51]. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

The extremely low picomolar dissociation constants for the G-protein-GTP complexes imply that finding molecules that can effectively compete with and displace the GTP from the binding sites has been a huge challenge [65]. Polyisoprenylation pathway modifications are important not only for functional localization but also for the protein-protein interactions that govern their translocation, additional functional regulation and stability to proteases [66, 67]. The methyltransferase activity that generates the carboxyl methyl esters on the terminal polyisoprenylated cysteine is countered by PMPMEase that hydrolyzes the methyl ester substrates (Fig. 1). This has been demonstrated using small molecule polyisoprenylated substrates [14, 15, 37, 42] and RhoA [68].

3.5. Polyisoprenylation-dependent Protein-protein Interactions With Chaperone Proteins

Adding to the complexity by which the monomeric G-proteins are regulated, are studies showing their complexation with other proteins in a polyisoprenylation-dependent manner. These include proteins such as Rho-GDIs, polyisoprenylated Rho acceptor protein 1 (PRA1), calmodulin (CaM) and Galectin 3. Rho-GDIs, PRA1, 14-3-3 proteins and CaM are reported to solubilize the polyisoprenylated proteins from cell membranes into the cytoplasm, thereby diminishing the interactions with cell surface receptors required for their activation. Various studies have demonstrated that interactions of the different G-proteins with these chaperone

one proteins as well as the shuttling back and forth to the plasma membrane, are dynamic [69, 70]. It is unclear at this moment whether the PCAIs have any effects on the interactions of G-proteins with these chaperone proteins. If so, proteins with the polyisoprenyl-binding pockets would constitute targets for the PCAIs (Fig. 4). If that were the case, it would be interesting to know what the consequences of PCAIs treatment on (1) RTKs signaling, (2) effector interactions with the polyisoprenylated proteins, (3) proteolytic stability of the affected G-proteins and (4) the cellular responses, and which of these cellular responses would contravene cancer progression (Fig. 5).

3.6. Effects of PCAIs on Signaling Pathways

RTKs overexpression and/or gain in function mutations contribute to the progression of various neoplasms (Table 2). To mitigate the excessive signaling, monoclonal antibody (MABs) biologics are directed at the extracellular domains of the RTKs and/or their ligands to suppress signaling. Kinase inhibitors such as erlotinib target the intracellular mutated tyrosine kinase domains that drive some cancers. These targeted therapies are, however, ineffective in cancers that harbor tumor-promoting changes in downstream mediators such as Ras [88, 89]. The PCAIs targeting of polyisoprenylated protein interactomes is potentially beneficial at treating tumors that harbor G-protein hyperactivities as well as those of RTKs and other upstream drivers (Fig. 6).

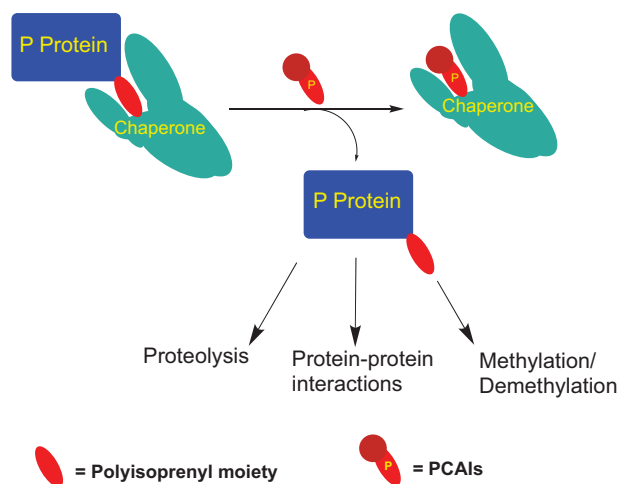


Fig (5). Possible interactions of PCAIs with polyisoprenylated protein chaperones. PCAIs may competitively disrupt the well-defined polyisoprenylation-dependent interactions of polyisoprenylated proteins (P protein) with chaperone proteins [16], resulting in their dislodgement such that their localizations, functions, biotransformation and breakdown patterns become altered. This is exemplified by the phosphorylation of the hypervariable region of K-Ras4B that is inhibited by its binding to CaM, thereby controlling its polyisoprenylation-dependent interactions [87] and depletion of K-Ras [50], RhoA, Rac1 and Cdc42 [48]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Some prominent growth factors and receptor tyrosine kinases rely on monomeric G-proteins such as Ras and Rho to drive cancer progression. Using the PCAIs to uncouple the functional protein-protein interactions involving G-proteins downstream of the receptors might curtail the excessive signaling from the upstream receptors as K-Ras, Rho, Rac1 and Cdc42 that drive cancer progression are depleted in PCAIs-treated cancer cells [48, 50, 51, 71].

| Protein | Cancer type | Aberrations | Incidence rate (%) | References |
|---|----------------------------|----------------------------|--------------------|------------|
| Epidermal growth factor receptor, EGFR (ErbB-1) | Pancreatic cancer | Overexpression | 90 | [72] |
| | Glial tumors | Overexpression | 50 | [73] |
| | Non-small cell lung cancer | Amplification and mutation | 10 | [73, 74] |
| | Non-small cell lung cancer | Overexpression | 40-80 | [75] |
| | Breast | Mutation | 60 | [53] |
| | Head and Neck | Amplification | 7 | [76] |
| | Colon cancer | Amplification | 6 | [77] |
| | Ovarian cancer | Overexpression | 64 | [78] |
| HER2 (ErbB-2) | Breast cancers | Overexpression | 20-25 | [79] |
| | Colon cancer | Overexpression | 15.5 | [80] |
| ErbB-3 | Lung | Overexpression | 50-70 | [81] |
| | Breast | Overexpression | 50-70 | [81] |
| | Colon | Overexpression | 50-70 | [81] |
| ErbB-4 | Colon | Overexpression | 22 | [81] |
| IGF1R | Breast | Amplification | 10 | [81] |
| | Melanoma | Amplification | 3 | [81] |
| | Prostate cancer risk | Amplification | 4.3 | [82] |
| IGF2R | Breast | Mutations | 40 | [83] |
| | Hepatocellular | Mutations | 80 | [84] |
| | Colorectal | Mutations | 5 | [85] |
| bFGF | Prostate | Overexpressed | 83 | [86] |
| VEGF | Colon | Overexpression | 55.5 | [80] |
| | Ovarian cancer | Overexpression | 25 | [78] |

The Ras-Raf-MEK-ERK signaling cascade appears to be susceptible to PCAIs treatment as K-Ras has been demonstrated to be depleted while ERK 1/2 phosphorylation was stimulated 48 h after treatment with PCAIs [50]. It is uncertain at this moment whether the depletion of these monomeric G-proteins is due to suppression of their synthesis or that PCAIs-induced dissociation from their chaperones leaves them exposed and vulnerable to proteolytic degradation. The depletion of K-Ras following treatment with the PCAIs is consistent with its role as an oncogenic protein and the apoptotic effects of the PCAIs on cancer cells harboring wild type as well as the mutant constitutively active K-Ras [50]. Although the stimulation of ERK 1/2 phosphorylation is counter to expectations given the PCAIs-induced depletion of K-Ras, numerous studies have linked increased ERK activity to the extrinsic apoptotic pathway involving caspase-8 activation (reviewed in [90]).

We have so far shown that the PCAIs induce apoptotic cell death in cancer cells harboring K-Ras mutations from pancreatic [24] and hyperactive EGFR in prostate cancer [47, 49]. They inhibit cell migration by disrupting F-actin organization, filopodia and lamellipodia, resulting in cell rounding. The effects on F-actin, leading to inhibited cell migration, also result in the inhibition of tube formation in human umbilical vein endothelial cells (HUVEC) assays for angiogenesis [46]. In *in vivo* analysis using chick chorioallantoic membrane and zebrafish embryos, the PCAIs inhibited angiogenesis – like phenomena at low micromolar concentrations [46]. These effects may be due in no small part to their effects on the monomeric G-proteins that regulate the cytoskeleton and cell motility. This was shown in our recent work in which treatment of cells with PCAIs resulted in significant depletion of RhoA, Cdc42 and Rac1 [48]. RhoA regulates focal adhesion, a critical event in cell migration, invasion and angioge-

nesis [44, 45, 91]. Its depletion somewhat explains the observed strong inhibitory effects of the PCAIs on cell migration and invasion [47, 48] as well as angiogenesis [46] that rely on focal adhesion formation to anchor cells to the extracellular matrix for traction.

Rac1 and Cdc42 are monomeric G-proteins that also play critical roles in the formation of filopodia and lamellipodia at the leading edges of migrating cells [44]. This effect was compounded by the fact that the levels of integrin $\alpha 4$ were suppressed by over 50% upon treatment with PCAIs. The levels of cleaved integrin $\alpha 4$ increased correspondingly [51]. The depletion of these proteins is therefore consistent with the antian-

giogenic and anti-invasive nature of the PCAIs that underscores a potential anti-metastatic effect.

3.7. Effect of the PCAIs on Focal Adhesion Proteins

In addition to the depletion of the polyisoprenylated proteins RhoA, Rac1 and Cdc42, the PCAIs also induce the depletion of vinculin and fascin in treated cells as observed by western blotting [51]. Furthermore, vinculin depletion correlated with almost complete dissipation of vinculin punctates [51]. The intracellular immunofluorescence intensity of fascin, an actin-binding protein, decreased with the concentration of PCAIs treatment. The levels of other proteins such as α -actinin and integrin $\beta 5$ were unchanged.

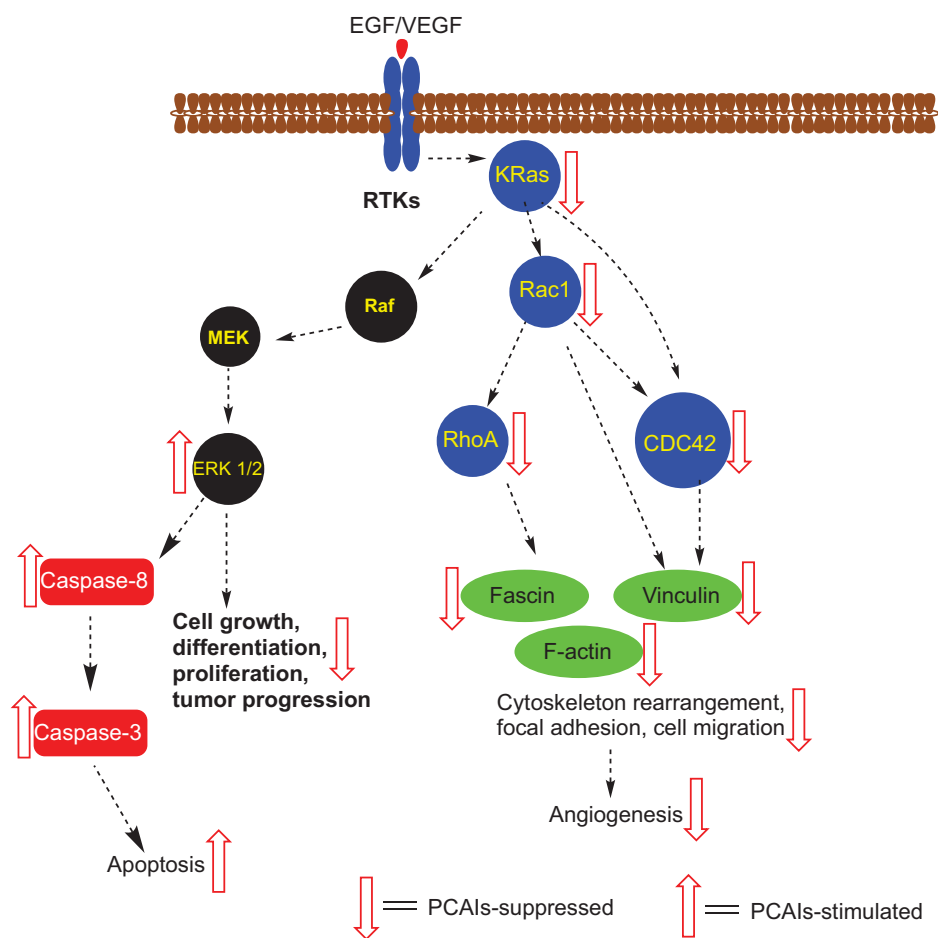


Fig. (6). Cellular processes inhibited by the PCAIs suggest interactions with receptor tyrosine kinase (RTKs) signal transduction pathway intermediates. Polyisoprenylated proteins (blue circles) are the most likely direct targets of the PCAIs as they are depleted from cancer cells treated with the analogs. Fascin and vinculin that contribute to the formation of focal adhesion complexes are also depleted. In combination with the inhibition of F-actin, processes that depend on the filamentous actin cytoskeleton and focal adhesions, such as cell migration and angiogenesis, are largely abolished at low to submicromolar concentrations of the PCAIs. Cancers with such drivers as the growth factors EGF and VEGF and the RTKs, which occur upstream of the polyisoprenylated proteins, are also prone to inhibition by virtue of their dependence on the G-proteins as signaling mediators. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

CONCLUSION AND FUTURE DIRECTIONS

Although the initial objective for the design and synthesis of the PCAIs was to inhibit PMPMEase and control its hyperactivities that spur cancer cell survival and proliferation, their poor inhibitory potencies against the enzyme (K_i values of 3.7 to 20 μM) compared to their superior activities against cancer cell viability was a strong indication that the latter effects are unlikely to be as a result of PMPMEase inhibition. However, the effects against cancer cell growth, proliferation, colony formation, migration and invasion, angiogenesis and disruption of F-actin organization are consistent with perturbations in polyisoprenylated protein function given the intricate signaling roles that Ras and Rho protein families play in these cellular events. The PCAIs have been demonstrated to be effective against cancer cells with hyperactive epidermal growth factor receptors (EGFR, RTKs) and constitutively active mutant K-Ras on which kinase inhibitors such as erlotinib are ineffective [88, 89]. In addition to their ability to disrupt the F-actin cytoskeleton, cell migration and invasion, as well as their inhibition of angiogenesis, implies that the PCAIs may have a multifaceted mechanism against cancer progression. They potentially could have broad clinical applications for treating cancers with hyperactive growth factors, RTKs and G-proteins such as Rho and Ras. Synthetic optimization to improve potency, pharmacological target identification, bioavailability and *in vivo* testing will contribute to their continuous development into a novel class of targeted agents to address the unmet therapeutic need for neoplasms involving hyperactive monomeric G-protein signaling pathways.

LIST OF ABBREVIATIONS

| | | | |
|-------|--|--------------|---|
| AML | = Acute Myelogenous Leukemia | GEF | = Guanine Nucleotide Exchange Factor |
| bFGF | = Basic Fibroblast Growth Factor | GF | = Growth Factors |
| CaM | = Calmodulin | GTP | = Guanosine Triphosphate |
| CAM | = Chorioallantoic Membrane | GTPase | = Guanosine Triphosphate Hydrolase |
| Cdc42 | = Cell Division Control Protein 42 | hCE1 | = Human Carboxylesterase 1 |
| CES1 | = Carboxylesterase 1 or PMPMEase | HER2 | = Human Epidermal Growth Factor Receptor Type 2 |
| EGFR | = Epidermal Growth Factor Receptor | H-Ras | = Harvey Rat Sarcoma |
| ERK | = Extracellular Signal-regulated Kinase or Mitogen-activated Protein Kinase (MAPK) | HUVEC | = Human Umbelical Vein Endothelial Cell |
| GAP | = GTPase-activating Protein | IGF1R | = Insulin-like Growth Factor 1 Receptor |
| GDP | = Guanosine Diphosphate | IGF2R | = Insulin-like Growth Factor 2 Receptor |
| | | K-Ras | = Kirsten Rat Sarcoma |
| | | MAB | = Monoclonal Antibody |
| | | MEK MAPK/ERK | = Kinase or Mitogen-activated Protein Kinase Kinase (MAPKK) |
| | | N-Ras | = Neuroblastoma Rat Sarcoma |
| | | PCAI | = Polyisoprenylated Cysteiny Amide Inhibitors |
| | | PMPMEase | = Polyisoprenylated Methylated Protein Methyl Esterase |
| | | PMSF | = Phenylmethylsulfonyl Fluoride |
| | | PPMTase | = Polyisoprenylated Protein Methyl Transferase or Isoprenylcysteine Methyl Transferase (ICMT) |
| | | PRA1 | = Polyisoprenylated Rho Acceptor Protein 1 |
| | | RAC1 | = Ras-related C3 Botulinum Toxin Substrate 1 |
| | | RAC2 | = Ras-related C3 Botulinum Toxin Substrate 2 |
| | | Raf | = Rapidly Accelerated Fibrosarcoma or Mitogen-activated Protein Kinase Kinase Kinase (MAPKKK) |
| | | RCE1 | = Ras Converting Enzyme 1 |
| | | RhoA | = Ras Homolog Family Member A |
| | | Rho-GDIs | = Rho-dissociation Inhibitors |

RTKs = Receptor Tyrosine Kinases
 SAM = S-adenosyl-L-methionine
 VEGF = Vascular Endothelial Growth Factor

CONSENT FOR PUBLICATION

Not Applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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