Published in final edited form as:

Bioorg Med Chem. 2012 January 1; 20(1): 283–295. doi:10.1016/j.bmc.2011.10.087.

Amide-Modified Prenylcysteine based Icmt Inhibitors: Structure Activity Relationships, Kinetic Analysis and Cellular Characterization

Jaimeen D. Majmudar^{1,3}, Heather B. Hodges-Loaiza³, Kalub Hahne³, James L. Donelson^{1,3}, Jiao Song^{1,3}, Liza Shrestha^{1,3}, Marietta L. Harrison^{1,3}, Christine A. Hrycyna^{*,3}, and Richard A. Gibbs^{*,1,3}

¹Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, 47907

³Department of Chemistry and The Purdue University Center for Cancer Research, Purdue University, West Lafayette, IN, 47907

Abstract

Human protein isoprenylcysteine carboxyl methyltransferase (hIcmt) is the enzyme responsible for the α -carboxyl methylation of the C-terminal isoprenylated cysteine of CaaX proteins, including Ras proteins. This specific posttranslational methylation event has been shown to be important for cellular transformation by oncogenic Ras isoforms. This finding led to interest in hIcmt inhibitors as potential anti-cancer agents. Previous analog studies based on *N*-acetyl-*S*-farnesylcysteine identified two prenylcysteine-based low micromolar inhibitors (**1a** and **1b**) of hIcmt, each bearing a phenoxyphenyl amide modification. In this study, a focused library of analogs of **1a** and **1b** was synthesized and screened versus hIcmt, delineating structural features important for inhibition. Kinetic characterization of the most potent analogs **1a** and **1b** established that both inhibitors exhibited mixed-mode inhibition and that the competitive component predominated. Using the Cheng – Prusoff method, the K_i values were determined from the IC_{50} values. Analog **1a** has a K_{IC} of $1.4 \pm 0.2 \mu M$ and a K_{IU} of $4.8 \pm 0.5 \mu M$ while **1b** has a K_{IC} of $0.5 \pm 0.07 \mu M$ and a K_{IU} of $1.9 \pm 0.2 \mu M$. Cellular evaluation of **1b** revealed that it alters the subcellular localization of GFP-KRas, and also inhibits both Ras activation and Erk phosphorylation in Jurkat cells.

Keywords

Isoprenylcysteine carboxylmethyltransferase; methylesterification; Icmt; enzyme inhibition; Ras proteins; anti-cancer agents; prenylation; methyl transferase

1.0 Introduction

A target of growing interest in the field of cancer chemotherapeutics is the human protein isoprenylcysteine carboxyl methyltransferase (hIcmt).^{1, 2, 3, 4} Icmt is the enzyme responsible

© 2011 Elsevier Ltd. All rights reserved.

*Address correspondence to: rgibbs@purdue.edu, hrycyna@purdue.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

for the last common step of the posttranslational processing pathway of CaaX proteins.^{5, 6} The family of CaaX proteins includes many key eukaryotic regulatory proteins, most notably proteins in the Ras family. These proteins terminate with a C-terminal CaaX sequence that is composed of four amino acids in the order of cysteine, followed by two generally aliphatic residues (aa), and a final residue (X) that varies in its identity from protein to protein. The first step of the CaaX protein posttranslational processing pathway is mediated by either protein farnesyltransferase (FTase) or protein geranylgeranyltransferase type I (GGTase-I).⁷⁻⁹ FTase and GGTase-I respectively attach either a 15-carbon or 20-carbon isoprenoid group to the CaaX cysteine. The type of lipid group added is directed, in part, by the nature of the terminal X residue. Lipidation is followed by proteolytic removal of the -aaX residues by the endoprotease Ras converting enzyme-1 (Rce1).¹⁰ Icmt then carries out the final carboxyl methylation step. Disruption of this pathway via enzyme inhibition prevents Ras proteins from localizing properly in the cell, thereby hindering cellular functionality and obstructing cellular growth.^{2, 11, 12} Therefore, the development of inhibitors of the enzymes in this pathway is desirable for the treatment of cancer driven by oncogenic Ras.

Mutant forms of K-Ras are a frequent cause of several human malignancies, including pancreatic cancers. Research on farnesyltransferase inhibitors (FTIs), once promising cancer chemotherapeutic agents, showed that K-Ras tumors are often resistant to FTI treatment due to alternative geranylgeranylation.¹³⁻¹⁶ The reason for this difference lies in the fact that K-Ras can be alternatively geranylgeranylated by GGTase I in the presence of FTIs.¹⁷⁻¹⁹ Although, Icmt recognizes both farnesylated and geranylgeranylated proteins,^{20, 21} previous studies suggest that the methylation is more important for the proper localization of farnesylated CaaX proteins than for many geranylgeranylated proteins.^{22, 23}

These data further support that Icmt may prove to be an important target in preventing the function of farnesylated oncogenic Ras proteins, but may not be detrimental to the function of all CaaX proteins. Furthermore, recent studies in cells and mouse models also support Icmt as a promising chemotherapeutic target.^{24, 25} Wang *et al.* have shown that small molecule Icmt inhibitors can induce autophagic cell death in PC3 prostate cancer cells.²⁴ In mice, inactivation of Icmt has also been shown to inhibit transformation by oncogenic K-Ras and B-Raf.² These key findings further support the development of hIcmt inhibitors as chemotherapeutic agents for Ras-driven cancers.

Our laboratories have recently identified 2-phenoxy-phenylfarnesylcysteine (POP-FC, Figure 1a) and 2-phenoxyphenyl-3-methylbutenyl-farnesylcysteine (POP-3-MB-FC, Figure 1b) as lead Icmt inhibitors.²⁶ These findings prompted us to examine the role of the phenoxy-phenyl motif in achieving Icmt inhibition. The simplest and most widely used amino acid-based Icmt substrate is an amide-modified farnesylated cysteine, *N*-acetyl-*S*-farnesylcysteine (AFC).²⁷⁻³³ The important substrate recognition features of AFC, which mimics the -aaX proteolyzed C-terminus of protein substrates, are the presence of an *S*-farnesyl moiety, a free α -carboxyl group and an amide modified α -amino group.^{27, 29} Note that the peptide Icmt substrate LARYK-*S*-farnesyl-C was found to have a 10-fold lower K_M for mammalian Icmt than AFC,³³ suggesting that the upstream sequence plays a role in recognition and methylation efficiency.

Previous studies from our laboratory have indicated that the human homolog of Icmt may possess an active site that can accommodate substrates or inhibitors with bulky, hydrophobic amide moieties and that certain bulky amide-modified farnesylcysteine (AMFC) analogs can act as low micromolar inhibitors of human Icmt.^{26, 34, 35} In order to further our understanding of the role the phenoxy-phenyl motif plays in human Icmt (hIcmt) inhibition, a library of compounds was designed and synthesized based on the lead POP-FC (**1a**).

Results from this study will help us better understand the role of the phenoxy-phenyl motif in enzyme recognition with the goal of enhancing the potency of these small molecules.

2.0 Chemistry

The analogs described herein were synthesized using previously established methods in our laboratory.^{26, 35} The general synthetic design involved utilizing a known synthesis of farnesylcysteine methyl ester³⁵ and coupling it with the appropriate carboxylic acid using HOBt and HBTU followed by saponification using lithium hydroxide (as shown schematically in Scheme 2).³⁵ In cases where the carboxylic acid was not commercially available, the appropriate aromatic carboxylic acid was synthesized and coupled to farnesylcysteine methylester.

The structures of the compounds synthesized utilizing this approach are shown in Figure 2. Carboxylic acids needed for the synthesis for compounds **2a-c** and **2e** were commercially available. For compounds **2d** and **2f-h**, each carboxylic acid was synthesized as shown in Scheme 1. The general method involved using an alkoxide anion as the nucleophile to displace a benzylic halide to obtain the respective phenoxy-phenyl analog. The aromatic bromide was then converted into a Grignard reagent using magnesium turnings in THF and quenching the formed Grignard with dry ice. Carboxylic acid **5** used to prepare analog **2d** was synthesized using an Ullmann-type ether coupling as reported previously.³⁶

We also designed and synthesized molecules in which the phenoxy phenyl amide scaffold was maintained but the prenyl chain was modified. To this end, we synthesized analogs **17-21** shown in Figure 3. Their synthesis is depicted in Schemes 3 and 4. Analog **17** is the enantiomer of our lead **1a**, while analogs **18** and **19** contain a shorter geranyl (10 carbon) and a longer geranylgeranyl (20 carbon) chain, respectively. Analog **20** (synthesis shown in Scheme 4) was specifically designed to investigate the role of the amide proton in hIcmt inhibition and analog **21**, with an undecyl moiety was designed to probe the importance of the prenyl chain in achieving hIcmt inhibition. We have recently reported that a similar change from prenyl to straight chain alkyl is detrimental to hIcmt inhibition when the amide linkage was replaced with a sulfonamide bond.³⁷ We wanted to investigate whether such a change is consistent across different classes of inhibitors. The *N*-methyl analog **20** was synthesized using a solid-phase methodology using the advanced intermediate **23**, which was synthesized as reported previously.³⁸ Compound **23** was loaded on the 2-chlorotrityl-chloride resin, which is essential to this synthesis due to the acid sensitivity of the prenyl chain. After loading, the disulfide-protecting group was removed using DTT and the farnesyl group was attached to the free thiol using farnesyl chloride. The 2-phenoxyphenyl carboxylic acid was then coupled to the secondary amine using standard coupling procedures previously used in our laboratory.²⁶ Compound **20** was obtained upon cleavage from the resin using 0.2% TFA in DCM. Following their syntheses, the compounds were evaluated as substrates and inhibitors of hIcmt using a vapor diffusion assay.^{5, 39, 40}

3.0 Results and Discussion

3.1 *In vitro* Biochemical Evaluation of Analogs

Analog **2a-h** and **17-21** were first tested as substrates for hIcmt at 25 μ M as described in the experimental section. Upon evaluation, none of the analogs showed substrate activity (data not shown). The compounds were subsequently tested as inhibitors at 10 μ M in the presence of 25 μ M of the substrate AFC and all the analogs were inhibitors of hIcmt to varying degrees. Compounds **2a** and **2b** were specifically synthesized and tested to evaluate the importance of the positioning of the phenoxy-phenyl motif in hIcmt inhibition. Both of these analogs were poor inhibitors of hIcmt, both inhibiting hIcmt by less than 30% at 10

μM . We experimentally determined the IC_{50} of compound **2b** to be $22.6 \pm 1.2 \mu\text{M}$. This value reflects an approximately five-fold loss in activity between the *ortho* and *para* isomers of the phenoxy-phenyl motif, suggesting that the *ortho*-phenoxyphenyl motif is important for hIcmt inhibition. Note that the *para* regio-isomer is a significantly poorer inhibitor compared to both the *meta* and the *ortho* isomer.

We have previously reported the synthesis of the anthranilic acid analog (Figure 1c), where the phenoxy-phenyl oxygen is replaced by a nitrogen atom.²⁶ This compound has an IC_{50} of $7.1 \mu\text{M}$ against hIcmt. Although less potent than the parent compound **1a**, the less electronegative nitrogen does not result in a significant loss of activity. We next wanted to evaluate the effect of changing the size and electronegativity of the central atom connecting the *ortho*-substituted phenyl ring. To this end, we synthesized analogs **2c** and **2d**. The decreased electronegativity of the carbon and sulfur atom linkers resulted in a roughly four-fold loss of inhibitory activity as compared to compound **1a**, suggesting that the electronegativity of the connecting atom between the two-phenyl rings may be a determining factor for inhibitory activity. More electronegative atoms such as oxygen and nitrogen present in the structure resulted in better inhibitors than those containing less electronegative atoms such as carbon and sulfur.

The biochemical evaluation described above suggested that the *ortho* substituted phenoxy-phenyl motif was important for hIcmt inhibition. To further investigate the effect of the spatial orientation of the oxygen connector atom and the second phenyl ring on hIcmt inhibition, we synthesized and evaluated analogs **2e-2h**. Conformational restriction of the two phenyl rings through a dibenzofuran scaffold (compound **2e**) diminished inhibitory activity significantly, as did replacing the central one oxygen linker with a two-atom linker in analogs **2f** and **2g**. It is worth noting that analog **2g**, which retains the position of the oxygen atom relative to the amide bond, displays much greater inhibition as compared to analog **2f**. The inhibitory effect of analog **1a** could result from the two oxygen atoms' (the amide carbonyl oxygen and the linker oxygen), involvement in a critical interaction because increasing the distance between those two oxygen atoms reduced the inhibitory potency of the molecule. Analog **2h**, where the central oxygen linker is more flexible compared to **1a** is a poor inhibitor compared to compound **1a**.

To evaluate the effect of the stereochemistry of the amino acid derivative POP (**1a**) on hIcmt inhibition, we synthesized its enantiomer (**17**). We have recently demonstrated that the stereochemistry at the alpha carbon is not critical for hIcmt inhibition in a sulfonamide series³⁷ and consistent with this result, analog **17** inhibited hIcmt with an IC_{50} of $7.8 \pm 0.4 \mu\text{M}$, nearly equivalent to **1a**, which demonstrated an IC_{50} of $6.2 \pm 0.7 \mu\text{M}$. Next, to interrogate the importance of the farnesyl chain in analog **1a**, we synthesized analogs **18**, **19** and **21**. The farnesyl group appears to be very important to hIcmt inhibition because the short and longer prenyl analogs **18** and **19** exhibited significant loss of hIcmt inhibition at $10 \mu\text{M}$. The undecyl analog, **21**, was a particularly poor inhibitor of hIcmt at the test concentration. These data suggest that the presence of a farnesyl chain on the cysteine sulfur is a critical pharmacophore for hIcmt inhibition. These data corroborate our previous findings about the importance of the farnesyl chain for hIcmt inhibition.^{34, 37} Finally, to interrogate the effect of the amide proton on hIcmt inhibition, we synthesized analog **20** using the solid-phase strategy described above. This analog was equivalent to our previous lead, **1a**, in terms of percent inhibition of hIcmt activity and has a similar IC_{50} of $6.7 \pm 0.4 \mu\text{M}$. The presence of a methyl group on the amide nitrogen appears to have minimal effect on the inhibition potential of the analog, suggesting that the amide region of the molecule may be no more important than serving as a linker between the phenoxy-phenyl and the farnesyl moieties.

Overall, our biochemical data suggest that the *ortho*-phenoxyphenyl motif is important and replacement with other regioisomers leads to a significant decrease in potency. The presence and the position of the oxygen atom connecting the two phenyl rings are also important features, as any change in the atom itself or its spatial positioning reduces hIcmt inhibition. Stereochemistry about the alpha carbon of cysteine and the amide proton both appear inconsequential to hIcmt inhibition. The most important element in hIcmt inhibition with this class of compounds is the farnesyl chain itself, as any deviation from the 15-carbon isoprene results in a loss of inhibition, which is particularly evident with the undecyl analog **21**, a very poor hIcmt inhibitor.

3.2 Kinetic Mode of Inhibition for Compounds **1a** and **1b**

In order to determine the mode of inhibition of hIcmt by compounds **1a** and **1b**, the most potent inhibitors in this series of compounds, kinetic assays were performed in which the small molecule substrate (AFC) concentration was varied while the inhibitor concentration remained fixed. The data for each inhibitor concentration were averaged from three trials, and plotted as double reciprocal plots (Figures 4 and 5). These inhibitors displayed mixed-type inhibition in which the competitive component dominated, as demonstrated by an α value greater than 1.⁵³ K_i values for Compounds **1a** and **1b** were then determined from the IC_{50} values using the Cheng – Prusoff method as adapted by Cer *et al.*⁵⁴ For compound **1a**, the competitive (K_{IC}) and uncompetitive (K_{IU}) values were $1.4 \pm 0.2 \mu M$ and $4.8 \pm 0.5 \mu M$, respectively. For compound **1b**, the K_{IC} and K_{IU} values were $0.5 \pm 0.07 \mu M$ and $K_{IU} 1.9 \pm 0.2 \mu M$, respectively.

4.0 Cellular Effects of Icmt Inhibitor **1b**

4.1 Pop-3MB (**1b**) alters the subcellular localization of GFP-K-Ras in Jurkat T cells

Proper posttranslational processing is a prerequisite for Ras membrane localization and function. To determine the cellular effects of our most potent inhibitor **1b**, the membrane localization of fluorescently tagged K-Ras was visualized in the presence and absence of **1b**.^{41, 42} We transfected Jurkat T cells with GFP-K-Ras and treated the cells with delivery vehicle alone, the statin drug simvastatin (which blocks K-Ras prenylation), or **1b**. The cells were fixed and GFP-K-Ras localization was visualized using fluorescent microscopy. After 24 hours of treatment, GFP-K-Ras localization was categorized into three groups: normal plasma membrane localization, partial mislocalization, and complete mislocalization. This was determined via visual inspection of randomly selected fields containing 100 cells. Representative examples of normal, partial and complete mislocalization are shown in Figure 6b (determined as described in the Experimental section). The results demonstrated that $0.1 \mu M$ **1b** prevented approximately 35% protein membrane localization while treatment with $10 \mu M$ compound **1b** prevented 75% of membrane localization (Figures 6a and 6b). These data suggest that **1b** is taken up into mammalian cells and functions as an Icmt inhibitor, preventing K-Ras membrane localization. Surprisingly, the data indicate that significant cellular mislocalization of GFP-K-Ras is seen at compound levels that are significantly lower than the biochemical IC_{50} value for **1b**. While we are unclear as to the reason for this result, there are at least two possibilities. First, this compound may preferentially accumulate in Jurkat cells. Alternatively, this compound may, in addition to inhibiting Icmt, block GFP-K-Ras through another mechanism, possibly related to that proposed for Salirasib by Kloog and coworkers.⁴⁶⁻⁴⁸

4.2 Pop-3MB (**1b**) inhibits Erk phosphorylation and Ras activation in Jurkat T cells

Low-grade stimulation through the T-cell receptor (TCR) in Jurkat cells results in Ras dependent Erk activation.⁴³ We therefore utilized signaling assays with low grade TCR stimulation to evaluate the effects of **1b** on Erk and Ras activation. Cells were treated with

delivery vehicle (DMSO), simvastatin, or **1b**. After 24-hour vehicle or drug treatment, Jurkat cells were low-grade stimulated through the TCR with anti-CD-3 ϵ to activate the Ras-MAPK-Erk pathway. To test Ras activation, the stimulated cells were lysed and active GTP-bound Ras was precipitated from whole cell lysates using a GST-RBD (Ras Binding Domain) pulldown assay.⁴⁴ As shown in Figure 7b, compound **1b** decreased Erk activation approximately three-fold compared to the control. Furthermore, **1b** also inhibited Ras activation significantly as seen in Figure 7c. These data suggest that the alterations in TCR-dependent Erk phosphorylation and Ras activation in Jurkat T cells are due to the inhibition of Ras methylation by compound **1b**.

5.0 Conclusion

We have demonstrated that low-micromolar inhibitors of hIcmt alter the sub-cellular location of GFP-Ras and also have an effect in the downstream pathway. Inhibition of anti-CD-3 ϵ dependent Erk phosphorylation and Ras activation in Jurkat T cells by compound **1b** suggests that hIcmt inhibitors hold promise in treatment of K-Ras driven cancers. Our SAR data with the entire series of compounds reported here have provided insight into the potential pharmacophoric requirements for hIcmt inhibition. As there is yet no three-dimensional structural information for hIcmt, given that it is an integral membrane protein, these data generated from our compounds are a small step forward in understanding the inhibition requirements for hIcmt. Stereochemistry at the alpha carbon has no significant bearing on the outcome for inhibition. Our data further suggest that the farnesyl chain is the most important scaffold for substrate-based Icmt inhibitors and any change in length or overall hydrophobicity decreases inhibition. Cysmethynil, a small molecule inhibitor of Icmt⁴⁵ has demonstrated induction of autophagic cell death in PC3 prostate cancer cells,²⁴ and thus has shown promise for cancer therapy. Salirasib, a partial inhibitor of Icmt has also shown promise in phase I clinical trials.⁴⁶⁻⁴⁸ In contrast, a recent report demonstrated that very potent Icmt inhibitors reduced cell viability only modestly in several cancer cell lines.⁴⁹ These reports, coupled with our results published here suggest that Icmt inhibitors have utility in treating Ras-driven cancers. Further studies with substrate-based hIcmt inhibitors, such as those developed in our laboratory, will help to clarify the therapeutic potential of hIcmt inhibition. Analog **1b** is a promising lead and suggests that appropriate prenyl mimics can be developed to achieve potent hIcmt inhibition and efforts are currently underway to exploit these findings.

6.0 Experimental

6.1 Synthesis and Spectral Analysis

General Procedure for the synthesis of analogs **2a-2h**

Synthesis of farnesylcysteine methylester—L-cysteine methylester hydrochloride (1.0 eq.) (obtained from Sigma-Aldrich) was charged to a dry round bottom flask and the atmosphere inside the flask was replaced with argon. The reaction mixture was maintained at 0 °C and 5mL of 7 N ammonia in methanol (purchased from Sigma-Aldrich) was added to the L-cysteine methylester hydrochloride. The contents were allowed to stir for 15 min following which, *trans-trans*-farnesylchloride (0.95 eq.) (purchased from Sigma-Aldrich) was syringed into the flask. The reactants were allowed to react for 3 hours at 0 °C. The reaction was monitored by TLC analysis. The contents of the round bottom flask were evaporated to dryness and purified by column chromatography via isocratic elution using a mixture of methanol and dichloromethane (1:9). This yielded farnesylcysteine methylester in 80% yield. The spectral data matched the reported data.^{35, 50}

Synthesis of the amide-modified farnesylcysteine methylester intermediate—

The appropriate carboxylic acid (1.0 eq) was charged to a dry round bottom flask and was dissolved in dimethylformamide (DMF). Diisopropylethylamine (1.1 eq), HOBT (1.1 eq.) and HBTU (1.1 eq.) were added to this and the contents were allowed to react at 0 °C for 30 min. This was followed by addition of a solution of farnesylcysteine methylester in DMF. The contents were allowed to react for two hours. The reaction was monitored by TLC analysis. On completion of the reaction, 10% aqueous citric acid was added to the reaction mixture and the product was extracted using ethylacetate (20 mL) as the solvent. The solvent was removed under vacuum utilizing a rotary evaporator and the residue was loaded on a short (2-3 inches) silica gel plug. The silica gel plug was flushed with 3% methanol in dichloromethane to obtain the crude coupling product.

Synthesis of the amide-modified farnesylcysteine (free acid)—The crude product obtained from the previous step was dissolved in methanol and was transferred to a round-bottom flask. The contents were allowed to cool to 0 °C. Lithium hydroxide (1.1 eq.) was added to the mixture and the contents were allowed to react for two hours. Following the completion of the reaction, the solvent was removed under reduced pressure and the mixture was loaded on a silica gel column. Chromatographic separation was performed under the influence of gravity using a 1:9 mixture of methanol and methylenechloride.

Compound 2a: (*R*)-2-(4-phenoxybenzamido)-3-(((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)thio)propanoic acid—¹H NMR (300 MHz, CDCl₃) δ 7.80 (m, 3H), 7.33 (dd, *J* = 18.3, 10.6 Hz, 2H), 7.06 (m, 4H), 5.41 - 4.74 (m, 4H), 3.13 (m, 4H), 2.10 - 1.87 (m, 8H), 1.67 (d, *J* = 6.5 Hz, 3H), 1.59 (d, *J* = 4.5 Hz, 3H), 1.56 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 176.81, 135.09, 134.77, 131.17, 131.06, 129.86, 124.31, 124.03, 123.81, 119.74, 117.51, 39.66, 39.56, 26.69, 26.48, 25.70, 25.66, 25.63, 17.65, 17.59, 16.08, 15.95, 15.79.

Compound 2b: (*R*)-2-(3-phenoxybenzamido)-3-(((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)thio)propanoic acid—¹H NMR (300 MHz, CDCl₃) δ 8.03 - 7.68 (m, 4H), 7.67 - 7.46 (m, 3H), 7.37 (t, *J* = 7.2 Hz, 1H), 7.20 (d, *J* = 18.4 Hz, 1H), 5.38 (dd, *J* = 13.7, 7.3 Hz, 3H), 4.98 (d, *J* = 29.8 Hz, 1H), 3.61 - 2.94 (m, 4H), 2.48 - 2.08 (m, 8H), 1.94 (d, *J* = 14.1 Hz, 3H), 1.88 (s, 3H), 1.85 (s, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 176.38, 167.17, 157.37, 156.40, 139.73, 135.21, 135.05, 131.12, 129.73, 124.26, 124.22, 123.76, 123.57, 121.85, 121.50, 119.32, 119.07, 117.94, 53.49, 39.61, 39.51, 29.98, 26.64, 26.43, 25.62, 25.59, 17.61, 16.01, 15.91.

Compound 2c: (*R*)-2-(2-benzylbenzamido)-3-(((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)thio)propanoic acid—¹H NMR (300 MHz, MeOH) δ 7.32 (d, *J* = 6.7 Hz, 1H), 7.18 - 6.89 (m, 8H), 5.05 (t, *J* = 7.4 Hz, 1H), 4.92 (t, *J* = 6.3 Hz, 2H), 4.51 (s, 1H), 4.13 - 3.93 (m, 2H), 3.11 - 2.99 (m, 2H), 2.78 (ddd, *J* = 22.1, 13.6, 5.6 Hz, 2H), 1.98 - 1.72 (m, 8H), 1.50 (d, *J* = 4.1 Hz, 6H), 1.42 (s, 6H). ¹³C NMR (75 MHz, MeOH) δ 172.60, 160.82, 142.20, 140.57, 140.30, 137.60, 136.13, 132.03, 131.45, 131.01, 130.28, 129.36, 128.46, 127.10, 127.01, 125.43, 125.11, 121.51, 40.83, 40.71, 27.77, 27.42, 25.98, 17.85, 16.35, 16.21.

Compound 2d: (*R*)-2-(2-(phenylthio)benzamido)-3-(((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)thio)propanoic acid—¹H NMR (300 MHz, CDCl₃) δ 7.61 (d, *J* = 32.1 Hz, 1H), 7.37 (s, 3H), 7.28 (d, *J* = 3.6 Hz, 3H), 7.14 (s, 2H), 7.01 (s, 1H), 5.23 - 4.93 (m, 3H), 4.80 (s, 1H), 3.12 (s, 2H), 2.93 (t, *J* = 28.2 Hz, 2H), 1.99 (dd, *J* = 17.7, 6.9 Hz, 8H), 1.67 (s, 3H), 1.59 (s, 5H), 1.55 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 171.57, 168.04, 139.63, 136.60, 135.04, 133.98, 133.09, 131.15, 130.88, 129.32, 128.84,

127.92, 126.13, 124.27, 123.81, 119.51, 77.42, 77.00, 76.57, 58.58, 39.62, 39.53, 33.17, 30.02, 26.65, 26.46, 25.65, 17.64, 16.13, 15.95.

Compound 2e: (R)-2-(dibenzo[*b,d*]furan-4-carboxamido)-3-(((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)thio)propanoic acid—¹H NMR (300 MHz, CDCl₃) δ 8.56 (s, 1H), 8.09 (d, *J* = 17.2 Hz, 1H), 7.85 - 7.42 (m, 3H), 7.35 (s, 1H), 7.22 (s, 2H), 5.15 (s, 1H), 5.09 - 4.81 (m, 3H), 3.38 - 3.04 (m, 4H), 1.94 (dd, *J* = 19.0, 7.0 Hz, 8H), 1.64 (s, 3H), 1.56 (s, 3H), 1.47 (d, *J* = 12.5 Hz, 5H). ¹³C NMR (75 MHz, CDCl₃) δ 176.57, 168.75, 156.31, 152.11, 140.64, 136.04, 132.16, 129.75, 128.50, 125.29, 124.77, 124.21, 123.82, 121.35, 120.57, 112.87, 61.28, 40.60, 40.49, 34.29, 31.15, 27.65, 27.39, 26.64, 18.63, 17.00, 16.89, 12.33.

Compound 2f: (R)-2-(2-(phenoxymethyl)benzamido)-3-(((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)thio)propanoic acid—¹H NMR (300 MHz, CDCl₃) δ 7.61 (s, 1H), 7.47 (d, *J* = 6.6 Hz, 2H), 7.30 (d, *J* = 17.7 Hz, 1H), 7.21 (t, *J* = 7.0 Hz, 3H), 6.91 (dd, *J* = 20.5, 7.4 Hz, 3H), 5.34 - 4.92 (m, 5H), 4.61 (s, 1H), 2.97 (s, 3H), 2.62 (s, 1H), 2.10 - 1.79 (m, 8H), 1.68 (s, 3H), 1.60 (s, 3H), 1.55 (s, 3H), 1.51 (s, 3H).

Compound 2g—¹H NMR (300 MHz, CDCl₃) δ 8.72 (d, *J* = 7.2 Hz, 1H), 8.22 (dd, *J* = 7.8, 1.6 Hz, 1H), 7.50 (d, *J* = 6.5 Hz, 2H), 7.46 - 7.29 (m, 4H), 7.13 - 6.98 (m, 2H), 5.26 (d, *J* = 10.5 Hz, 2H), 5.10 (d, *J* = 18.0 Hz, 3H), 4.94 (dd, *J* = 12.1, 6.7 Hz, 1H), 3.08 (ddd, *J* = 20.4, 13.2, 7.8 Hz, 3H), 2.79 (ddd, *J* = 20.5, 13.9, 7.8 Hz, 2H), 2.05 - 1.90 (m, 8H), 1.67 (s, 3H), 1.62 - 1.57 (m, 9H). ¹³C NMR (75 MHz, CDCl₃) δ 175.33, 164.65, 156.96, 139.67, 135.43, 135.19, 133.04, 132.37, 131.20, 129.17, 128.49, 127.99, 124.33, 123.68, 121.39, 120.94, 119.57, 112.68, 77.43, 77.00, 76.58, 71.22, 52.44, 52.29, 39.61, 39.53, 32.90, 29.68, 26.63, 26.34, 25.62, 17.62, 16.01, 15.93.

Compound 2h: (R)-2-(2-((benzyloxy)methyl)benzamido)-3-(((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)thio)propanoic acid—¹H NMR (300 MHz, CDCl₃) δ 8.10 (d, *J* = 6.8 Hz, 1H), 7.71 (t, *J* = 8.2 Hz, 1H), 7.36 (dd, *J* = 11.4, 4.5 Hz, 2H), 7.33 - 7.29 (m, 4H), 7.27 (d, *J* = 3.7 Hz, 1H), 5.19 - 4.99 (m, 3H), 4.66 (d, *J* = 11.3 Hz, 1H), 3.36 (s, 4H), 3.07 (dd, *J* = 12.5, 7.4 Hz, 2H), 2.87 - 2.71 (m, 2H), 2.11 - 1.88 (m, 8H), 1.65 (d, *J* = 12.1 Hz, 3H), 1.58 (s, 6H), 1.56 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 175.72, 170.17, 140.64, 138.58, 136.17, 136.09, 132.14, 131.54, 131.21, 130.05, 129.28, 129.22, 128.65, 128.59, 125.21, 124.69, 120.37, 73.07, 71.12, 51.07, 40.57, 40.47, 34.25, 30.78, 27.60, 27.36, 26.58, 18.57, 16.97, 16.87.

Synthesis of Compound 5—To a solution of potassium hydroxide (5.0 eq.) in water were added 2-iodobenzoic acid (1.0 eq.), thiophenol (1.0 eq.) and Copper powder (0.1 eq.). The contents were refluxed for 12 hours, cooled to room temperature and acidified with 4 N hydrochloric acid. Acidification resulted in precipitation of product.

Compound 5: 2-(phenylthio)benzoic acid—¹H NMR (300 MHz, CDCl₃) δ 7.85 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.37 - 7.27 (m, 2H), 7.24 - 7.12 (m, 3H), 7.02 - 6.95 (m, 1H), 6.87 (dd, *J* = 10.9, 4.2 Hz, 1H), 6.52 (d, *J* = 8.1 Hz, 1H). ¹³C NMR (75 MHz, CDCl₃) δ 172.50, 145.62, 136.84, 134.13, 133.11, 133.00, 130.81, 130.30, 128.12, 126.19, 125.24.

General Synthetic methodology for bromides 9, 13 and 15—In general, phenol (1.0 eq.) (or the corresponding benzyl alcohol) was charged to a dry round-bottom flask and anhydrous DMF was added to it. The atmosphere in the flask was replaced with argon. Upon complete dissolution of the phenol (or the benzyl alcohol), the contents were cooled to 0 °C and allowed to stir for 15 min. This was followed by addition of sodium hydride (1.1 eq.)

and the compounds were allowed to react for one hour. Following this time period, a solution of the appropriate benzyl bromide (1.2 eq.) was added as a solution in anhydrous DMF over a period of 10 min. The contents were allowed to react for 3 hours. The reaction was quenched with ice and the product was extracted with ether, followed by washing with 10% citric acid (3×20 mL), then washed with 2M NaOH (to remove the unreacted phenol) and brine. The organic fractions were pooled and concentrated under vacuum to yield product. A chromatographic separation was performed using 2:8 ethylacetate and hexanes if it was necessary.

General Procedure for synthesis of carboxylic acids 10, 14 and 16—Magnesium turnings (4.0 eq.) were placed in a dry round bottom flask and flame-dried under a steady stream of argon. Upon cooling of the flask to room temperature, anhydrous THF and one drop of 1,2-dibromoethane were added to the flask. The contents were allowed to react for 5 min followed by addition of the appropriate bromide (as a solution in anhydrous THF) over a period of 5 min. The Grignard reagent was allowed to form for 2.5 hours and was quenched with dry ice. This was followed by addition of 10% hydrochloric acid (10 mL). The contents from the reaction quench were transferred to a separatory funnel, followed by addition of ethylacetate (20 mL). The aqueous phase was extracted with ethylacetate (3 × 20 mL). The combined organic extracts were treated with 5% sodium hydroxide (3 × 5 mL). Concentrated hydrochloric acid was added to the combined aqueous phase until the pH of the solution was 2.5, which resulted in the precipitation of the product. Filtration of the precipitate yielded the desired carboxylic acid in 45 – 78% yields.

Compound 9: 1-bromo-2-(phenoxy)methylbenzene—¹H NMR (300 MHz, CDCl₃) δ 7.71 (d, *J* = 8.0 Hz, 2H), 7.45 (dt, *J* = 7.6, 6.4 Hz, 3H), 7.29 (dd, *J* = 12.1, 4.4 Hz, 1H), 7.14 (t, *J* = 8.1 Hz, 3H), 5.27 (s, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 159.27, 137.20, 133.40, 130.41, 130.01, 129.69, 128.38, 123.11, 122.05, 115.71, 70.09.

Compound 10: 2-(phenoxy)methylbenzoic acid—¹H NMR (300 MHz, CDCl₃) δ 11.72 (s, 1H), 8.23 (d, *J* = 7.8 Hz, 1H), 7.87 (d, *J* = 7.8 Hz, 1H), 7.65 (s, 1H), 7.45 (s, 1H), 7.38 - 7.25 (m, 2H), 7.11 - 6.93 (m, 3H), 5.61 (s, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 173.87, 159.53, 141.79, 134.68, 132.73, 130.52, 130.48, 128.25, 127.24, 122.00, 115.93, 115.90, 69.02.

Compound 13: 1-(benzyloxy)-2-bromobenzene—¹H NMR (300 MHz, CDCl₃) δ 7.42 (dd, *J* = 7.8, 1.6 Hz, 1H), 7.33 (d, *J* = 7.3 Hz, 2H), 7.27 - 7.10 (m, 3H), 7.02 (dd, *J* = 7.8, 1.1 Hz, 1H), 6.76 - 6.61 (m, 2H), 4.90 (s, 2H). ¹³C NMR (75 MHz, CDCl₃) δ 155.71, 137.26, 134.12, 129.29, 129.20, 128.63, 127.73, 127.71, 122.85, 114.49, 71.29.

Compound 14: 2-(benzyloxy)benzoic acid—¹H NMR (300 MHz, CDCl₃) δ 10.52 (s, 1H), 7.77 (dt, *J* = 27.4, 13.7 Hz, 2H), 7.36 (d, *J* = 4.1 Hz, 1H), 7.32 (s, 2H), 7.00 (t, *J* = 7.5 Hz, 1H), 6.79 (ddd, *J* = 19.3, 17.3, 8.3 Hz, 3H), 5.15 (s, 2H).

Compound 15: 1-((benzyloxy)methyl)-2-bromobenzene—¹H NMR (300 MHz, CDCl₃) δ 7.62 - 7.49 (m, 2H), 7.49 - 7.27 (m, 6H), 7.23 - 7.08 (m, 1H), 4.69 (s, 4H). ¹³C NMR (75 MHz, CDCl₃) δ 138.97, 138.57, 133.43, 130.06, 129.84, 129.41, 129.37, 128.67, 128.33, 123.68, 73.67, 72.50.

Compound 16—¹H NMR (300 MHz, CDCl₃) δ 7.72 - 7.49 (m, 3H), 7.49 - 7.31 (m, 5H), 7.25 - 7.11 (m, 1H), 4.73 (s, 4H). ¹³C NMR (75 MHz, CDCl₃) δ 171.87, 137.97, 135.77, 133.43, 130.06, 129.84, 129.41, 129.37, 128.67, 127.45, 123.68, 72.58, 71.65.

Compound 17: (S)-2-(2-phenoxybenzamido)-3-(((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)thio)propanoic acid—¹H NMR (300 MHz, CDCl₃) δ 10.02 (bs, 1H), 8.77 (d, *J* = 6.7 Hz, 1H), 8.22 (d, *J* = 7.1 Hz, 1H), 7.35 (t, *J* = 7.9 Hz, 3H), 7.23 - 7.04 (m, 4H), 6.83 (d, *J* = 8.2 Hz, 1H), 5.07 (dd, *J* = 8.1, 3.9 Hz, 3H), 4.95 (d, *J* = 5.5 Hz, 1H), 3.18 - 2.86 (m, 4H), 1.99 (tt, *J* = 27.6, 13.8 Hz, 8H), 1.64 (d, *J* = 14.5 Hz, 3H), 1.58 (d, *J* = 4.3 Hz, 6H), 1.55 (s, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 174.65, 165.04, 155.79, 155.01, 139.55, 134.99, 132.96, 132.15, 131.03, 129.85, 124.49, 124.17, 123.67, 123.26, 122.50, 119.66, 119.41, 117.77, 52.82, 39.52, 39.40, 32.71, 29.74, 26.54, 26.27, 25.55, 17.54, 15.85.

Compound 18: (R,E)-3-((3,7-dimethylocta-2,6-dien-1-yl)thio)-2-(2-phenoxybenzamido)propanoic acid—¹H NMR (300 MHz, CDCl₃) δ 10.28 (s, 1H), 8.69 (d, *J* = 6.6 Hz, 1H), 8.20 (dd, *J* = 7.8, 1.7 Hz, 1H), 7.36 (dd, *J* = 10.7, 4.8 Hz, 3H), 7.16 (t, *J* = 7.4 Hz, 2H), 7.12 - 7.05 (m, 2H), 6.84 (d, *J* = 8.2 Hz, 1H), 5.10 - 4.98 (m, 2H), 4.91 (dd, *J* = 11.4, 6.0 Hz, 1H), 3.17 - 2.85 (m, 4H), 2.07 - 1.88 (m, 4H), 1.64 (s, 3H), 1.54 (d, *J* = 4.7 Hz, 6H). ¹³C NMR (75 MHz, CDCl₃) δ 174.74, 165.43, 156.12, 155.24, 139.95, 133.33, 132.39, 131.75, 130.15, 124.84, 123.98, 123.55, 122.66, 119.94, 119.64, 118.08, 52.89, 39.63, 32.63, 29.93, 26.51, 25.78, 17.79, 16.09.

Compound 19: (R)-2-(2-phenoxybenzamido)-3-(((2E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-yl)thio)propanoic acid—¹H NMR (500 MHz, CDCl₃) δ 9.73 - 9.72 (m, 1H), 8.85 (d, *J* = 6.8 Hz, 1H), 8.03 (dd, *J* = 7.8, 1.6 Hz, 1H), 7.46 - 7.32 (m, 3H), 7.18 (td, *J* = 7.6, 1.0 Hz, 2H), 7.12 - 7.05 (m, 2H), 6.89 - 6.82 (m, 1H), 5.16 - 4.98 (m, 4H), 4.68 (d, *J* = 4.8 Hz, 1H), 3.16 - 2.89 (m, 5H), 2.12 - 1.86 (m, 12H), 1.63 (d, *J* = 0.9 Hz, 3H), 1.61 - 1.49 (m, 12H). ¹³C NMR (126 MHz, CDCl₃) δ 177.26, 166.97, 157.50, 157.26, 140.42, 136.37, 136.08, 134.39, 132.73, 132.29, 131.40, 125.96, 125.70, 125.44, 125.36, 124.70, 121.87, 121.20, 119.63, 114.89, 50.10, 41.12, 41.05, 40.93, 34.66, 31.04, 28.07, 27.83, 27.68, 26.19, 18.06, 16.47, 16.43, 16.40.

Compound 20: (R)-2-(N-methyl-2-phenoxybenzamido)-3-(((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)thio)propanoic acid—¹H NMR (300 MHz, MeOH) δ 7.33 - 7.11 (m, 4H), 7.10 - 6.93 (m, 3H), 6.92 - 6.81 (m, 2H), 6.80 - 6.56 (m, 1H), 4.94 (d, *J* = 1.3 Hz, 3H), 4.30 - 4.13 (m, 1H), 3.12 - 2.98 (m, 1H), 2.94 (d, *J* = 7.6 Hz, 1H), 2.87 (d, *J* = 5.8 Hz, 1H), 2.81 (s, 2H), 2.72 (dd, *J* = 13.9, 4.2 Hz, 1H), 2.03 - 1.97 (m, 2H), 1.97 - 1.75 (m, 8H), 1.51 (s, 4H), 1.44 (s, 6H). ¹³C NMR (75 MHz, MeOH) δ 171.94, 157.82, 136.14, 132.05, 131.93, 130.91, 130.87, 125.44, 125.38, 125.13, 124.55, 121.60, 121.09, 120.98, 120.36, 119.12, 62.48, 40.84, 40.70, 30.69, 29.84, 27.78, 27.46, 25.97, 17.84, 16.31, 16.24, 16.20.

Compound 21: ((R)-2-(2-phenoxybenzamido)-3-(undecylthio)propanoic acid)—¹H NMR (300 MHz, MeOH) δ 7.93 (dd, *J* = 7.8, 1.7 Hz, 1H), 7.37 - 7.18 (m, 3H), 7.18 - 6.93 (m, 4H), 6.88 - 6.65 (m, 1H), 4.48 (t, *J* = 5.0 Hz, 1H), 2.94 (ddd, *J* = 33.3, 13.6, 5.0 Hz, 2H), 2.34 (dd, *J* = 10.9, 3.9 Hz, 2H), 1.39 - 1.23 (m, 2H), 1.12 (d, *J* = 17.7 Hz, 16H), 0.78 (t, *J* = 6.7 Hz, 3H). ¹³C NMR (75 MHz, MeOH) δ 178.93, 174.66, 166.37, 157.14, 134.02, 133.69, 132.52, 130.86, 125.56, 124.19, 121.04, 119.56, 37.67, 35.60, 33.33, 33.05, 30.97, 30.81, 30.74, 30.71, 30.49, 23.49, 14.39, 5.36.

6.2 Materials and Methods

Materials—*N*-2-Phenoxybenzoyl-*S*-3-(3-methyl-but-2-enyl)-5-(4-biphenyl)pent-2-enyl-L-cysteine (POP-3MB, compound **1b**) was synthesized in our laboratory²⁶ via established solution-phase methods and characterized to confirm identity and purity. Stock solutions were prepared at 50mM in DMSO and stored at -80 °C. EGFP-C3 vectors containing GFP

K-Ras was a generous gift from Mark Philips (New York University, School of Medicine). pGEX vector containing GST-RBD was a kind gift from Lawrence Quilliam (Indiana University School of Medicine). The following antibodies were used: Pan Ras (Oncogene, OP40), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2) Rabbit mAb (Cell Signaling Technology, 4377L), GAPDH (Ambion, 4300), CD-3e (UCHT1) (BD Pharmingen, 555329), ImmunoPure Goat Anti-Rabbit IgG, Peroxidase Conjugated (GARF, Pierce Biotechnology, 31462), ImmunoPure Goat Anti-Mouse IgG, Peroxidase Conjugated (GAMP, Pierce Biotechnology, 31432). Formaldehyde 37% solution was purchased from Mallinckrodt.

Cell culture—Jurkat T cells (E 6.1) were obtained and maintained as described previously.⁴² For all experiments, cells were treated using a vehicle (DMSO) or drug (diluted from a 50mM DMSO stock solution) at indicated concentrations for 24 h.

Fluorescence microscopy—Jurkat T cells (1.5×10^7 cells/500 μ l RPMI), transiently transfected with 20 μ g of either GFP-K-Ras construct (a kind gift from M. Phillips) using the Cell Porator (Life Technologies, 800 μ F, 250 V, low ohms), were treated with delivery group (DMSO), simvastatin alone (20 μ M), or various concentrations of POP-3MB (0.1, 1 or 10 μ M) for 24 hours in an incubator at 37 °C. The cells were harvested, washed with PBS three times, and plated on polylysine coverslips for 10 min. The coverslips were washed with PBS for 10 min (3 times) and the cells were fixed with 3% formaldehyde for 10 min. The coverslips were washed again for 10 min with PBS (3 times). After mounting the coverslips onto the slides using FluorSave™ Reagent (Calbiochem), the slides were dried for 45 min at room temperature. Images of cells were viewed using an Olympus BH-2 microscope and captured with a QImaging Microimager II. The captured images were viewed using Northern Eclipse software. Cells were classified as either full (fully mislocalized GFP-K-Ras construct with diffuse cellular staining), partial (partial proper localization), and normal (normal, primarily plasma membrane localization of GFP-K-Ras) by visual inspection.^{41, 42} The subcellular localization of GFP K-Ras was quantified using fluorescence microscopy (performed on an Olympus BH-2RFCA) as previously described.^{24, 51}

Isolation of Membrane Fraction from Yeast Cells—Membrane fractions from yeast cells were isolated as previously described.⁵ Crude membrane protein concentration was determined using Coomassie Plus Protein Assay Reagent (Pierce) according to the manufacturer's instructions, and compared with a bovine serum albumin standard curve.

Substrate and Inhibition Assays by In Vitro Vapor Diffusion Methyltransferase Assay—Methyltransferase assays were performed as previously described^{5, 39, 40} with minor modifications. The assay mixture contained a total volume of 60 μ L and a final Tris-HCl buffer concentration of 100 mM, pH 7.5. All reactions contained 20 μ M *S*-adenosyl-[¹⁴C-*methyl*]methionine (¹⁴C-SAM) and 5 μ g of membrane protein from His-hIcmt-overexpressing strain CH 2766.⁵ Initial substrate screening was performed at a single concentration of 25 μ M in duplicate, three times. For inhibition screening, protein and compound (10 μ M) were pre-incubated on ice in buffer, followed by the addition of 25 μ M AFC substrate and ¹⁴C-SAM. Reactions were incubated at 30 °C for 30 min and stopped by the addition of 50 μ L of 1 M NaOH/1% SDS. 100 μ L of this mixture was spotted on folded filter paper (6 cm x 2 cm) and lodged in the neck of a scintillation vial containing 10 mL of scintillation fluid, and capped to allow for diffusion of the released [¹⁴C]methanol into the scintillant. The filters were pulled out after 2.5 h, the vials tightly recapped and shaken, and then counted in a liquid scintillation analyzer.

Immunoblotting—Jurkat T cells ($3\text{--}5 \times 10^5$ cells mL⁻¹) were treated with vehicle (DMSO), simvastatin, or POP-3MB (1a). Cells were lysed in 1% Triton X-100 lysis buffer (25 mM Hepes, pH 7.2, 150 mM NaCl, 1% NP40, 5 mM EDTA, 10 µg mL⁻¹ of leupeptin and aprotinin, and 1 mM sodium vanadate), resolved on SDS-PAGE gels, resolved by SDS-PAGE on 12% gels and transferred to polyvinylidene difluoride membranes (Immun-Blot PVDF; Bio-Rad). Membranes were blocked with 5% goat serum and incubated with primary antibody (anti-Pan Ras (Oncogene OP40) for N/K Ras; or anti-GAPDH (Ambion 4300) for GAPDH) at room temperature for 1 h, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG. Membranes were developed using enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech) and autoradiography film. Quantitative western blotting was performed using ImageJ system.

Signaling Assay—Jurkat T cells (1×10^7 cells/1 ml) were washed in PBS, incubated with anti-CD-3e (20 µg/ml) for 15 min on ice, and stimulated at 37 °C for 10 min. Cells were washed and lysed in lysis buffer containing 1% Triton X-100, 20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 20 µg/ml each of leupeptin and aprotinin, 1 mM pyrophosphate, 1 mM glycerol phosphate, and 1 mM sodium orthovanadate. Samples were separated on 6-15% gradient SDS-polyacrylamide gels and analyzed by immunoblotting with anti-phosphoERK antibodies (at 4 °C overnight with anti-p-Erk (Cell Signaling Technology 4377L)). Membranes were developed using enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech) and autoradiography film. Quantitative western blotting was performed using ImageJ system.

GST-RBD Pull Down Assay—GST-RBD fusion protein was expressed in bacteria as previously described.⁵² After stimulation with anti CD3 antibody, Jurkat T cells (2×10^7) were lysed in 1% NP40 lysis buffer (1% Nonidet P-40, 25 mM HEPES, 150 mM NaCl, 0.25% sodium deoxycholate, 10% glycerol, 10 mM MgCl₂, 1 mM EDTA, 1 mM sodium vanadate, 10 mM NaF, 20 g/ml aprotinin and leupeptin, pH 7.4) and centrifuged at 464 x g for 3 min at 4 °C. The lysate was incubated with GST-RBD fusion protein conjugated glutathione-Sepharose 4B beads for 30 min at 4 °C. The resin was washed three times with Magnesium Lysis Buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate 10% glycerol, 10 mM MgCl₂), solubilized in 2X PSB (Protein Sample Buffer, Protein Sample Buffer = 100 mM Tris-Hcl, pH 6.8, 20% glycerol, 4% SDS, 0.2% bromophenol blue, 200 mM dithiothreitol) and the presence of activated Ras was detected by immunoblot analysis and quantified using the ImageJ system as mentioned above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Gelb MH, Brunsfeld L, Hrycyna CA, Michaelis S, Tamanoi F, Van Voorhis WC, Waldmann H. *Nat Chem Biol.* 2006; 2:518. [PubMed: 16983387]
2. Bergo MO, Gavino BJ, Hong C, Beigneux AP, McMahon M, Casey PJ, Young SG. *J Clin Invest.* 2004; 113:539. [PubMed: 14966563]
3. Winter-Vann AM, Casey PJ. *Nat Rev Cancer.* 2005; 5:405. [PubMed: 15864282]
4. Young SG, Clarke SG, Bergo MO, Phillips M, Fong LG. *The Enzymes.* 2006; 24:273.
5. Anderson JL, Frase H, Michaelis S, Hrycyna CA. *J Biol Chem.* 2005; 280:7336. [PubMed: 15611058]
6. Dai Q, Choy E, Chiu V, Romano J, Slivka SR, Steitz SA, Michaelis S, Philips MR. *J Biol Chem.* 1998; 273:15030. [PubMed: 9614111]
7. Clarke S. *Ann Rev Biochem.* 1992; 61:355. [PubMed: 1497315]

8. Zhang FL, Casey PJ. *Ann Rev Biochem.* 1996; 65:241. [PubMed: 8811180]
9. Basso AD, Kirschmeier P, Bishop WR. *J Lipid Res.* 2006; 47:15. [PubMed: 16278491]
10. Otto JC, Kim E, Young SG, Casey PJ. *J Biol Chem.* 1999; 274:8379. [PubMed: 10085068]
11. Bergo MO, Leung GK, Ambroziak P, Otto JC, Casey PJ, Gomes AQ, Seabra MC, Young SG. *J Biol Chem.* 2001; 276:5841. [PubMed: 11121396]
12. Bergo MO, Leung GK, Ambroziak P, Otto JC, Casey PJ, Young SG. *J Biol Chem.* 2000; 275:17605. [PubMed: 10747846]
13. Cohen SJ, Ho L, Ranganathan S, Abbruzzese JL, Alpaugh RK, Beard M, Lewis NL, McLaughlin S, Rogatko A, Perez-Ruixo JJ, Thistle AM, Verhaeghe T, Wang H, Weiner LM, Wright JJ, Hudes GR, Meropol NJ. *J Clin Oncol.* 2003; 21:1301. [PubMed: 12663718]
14. Macdonald JS, McCoy S, Whitehead RP, Iqbal S, Wade JL 3rd, Giguere JK, Abbruzzese JL. *Invest New Drugs.* 2005; 23:485. [PubMed: 16133800]
15. Van Cutsem E, van de Velde H, Karasek P, Oettle H, Vervenne WL, Szawlowski A, Schoffski P, Post S, Verslype C, Neumann H, Safran H, Humblet Y, Perez Ruixo J, Ma Y, Von Hoff D. *J Clin Oncol.* 2004; 22:1430. [PubMed: 15084616]
16. Whitehead RP, McCoy S, Macdonald JS, Rivkin SE, Neubauer MA, Dakhil SR, Lenz HJ, Tanaka MS, Abbruzzese JL. *Invest New Drugs.* 2006; 24:335. [PubMed: 16683076]
17. Whyte DB, Kirschmeier P, Hockenberry TN, Nunez-Oliva I, James L, Catino JJ, Bishop WR, Pai JK. *J Biol Chem.* 1997; 272:14459. [PubMed: 9162087]
18. Adjei AA. *J Natl Cancer Inst.* 2001; 93:1062. [PubMed: 11459867]
19. Rowell CA, Kowalczyk JJ, Lewis MD, Garcia AM. *J Biol Chem.* 1997; 272:14093. [PubMed: 9162034]
20. Michaelson D, Ali W, Chiu VK, Bergo M, Silletti J, Wright L, Young SG, Philips M. *Mol Biol Cell.* 2005; 16:1606. [PubMed: 15659645]
21. Volker C, Lane P, Kwee C, Johnson M, Stock J. *FEBS Letters.* 1991; 295:189. [PubMed: 1765152]
22. Michaelson D, Ali W, Chiu VK, Bergo M, Silletti J, Wright L, Young SG, Philips M. *Mol Biol Cell.* 2005; 16:1606. [PubMed: 15659645]
23. Roberts PJ, Mitin N, Keller PJ, Chenette EJ, Madigan JP, Currin RO, Cox AD, Wilson O, Kirschmeier P, Der CJ. *J Biol Chem.* 2008; 283:25150–25163. [PubMed: 18614539]
24. Wang M, Tan W, Zhou J, Leow J, Go M, Lee HS, Casey PJ. *J Biol Chem.* 2008; 283:18678–18684. [PubMed: 18434300]
25. Wahlstrom AM, Cutts BA, Liu M, Lindskog A, Karlsson C, Sjogren AK, Andersson KM, Young SG, Bergo MO. *Blood.* 2008; 112:1357–65. [PubMed: 18502828]
26. Donelson JL, Hodges-Loaiza HB, Henriksen BS, Hrycyna CA, Gibbs RA. *J Org Chem.* 2009; 74:2975. [PubMed: 19320430]
27. Tan EW, Perez-Sala D, Canada FJ, Rando RR. *J Biol Chem.* 1991; 266:10719. [PubMed: 1904056]
28. Volker C, Lane P, Kwee C, Johnson M, Stock J. *FEBS Lett.* 1991; 295:189. [PubMed: 1765152]
29. Volker C, Miller RA, McCleary WR, Rao A, Poenie M, Backer JM, Stock JB. *J Biol Chem.* 1991; 266:21515. [PubMed: 1939182]
30. Perez-Sala D, Gilbert BA, Tan EW, Rando RR. *Biochem J.* 1992; 284(Pt 3):835. [PubMed: 1622400]
31. Henriksen BS, Anderson JL, Hrycyna CA, Gibbs RA. *Bioorg Med Chem Lett.* 2005; 15:5080. [PubMed: 16183278]
32. Tan EW, Rando RR. *Biochemistry.* 1992; 31:5572. [PubMed: 1610803]
33. Stephenson RC, Clarke S. *J Biol Chem.* 1990; 265:16248. [PubMed: 2398053]
34. Anderson JL, Henriksen BS, Gibbs RA, Hrycyna CA. *J Biol Chem.* 2005; 280:29454. [PubMed: 15946942]
35. Donelson JL, Hodges HB, MacDougall DD, Henriksen BS, Hrycyna CA, Gibbs RA. *Bioorg Med Chem Lett.* 2006; 16:4420. [PubMed: 16777414]
36. Bonnet B, Soulez D, Girault S, Maes L, Landry V, Davioud-Charvet E, Sergheraert C. *Bioorg Med Chem.* 2000; 8:95. [PubMed: 10968268]

37. Majmudar JD, Hahne K, Hrycyna CA, Gibbs RA. *Bioorg Med Chem Lett*. 2011; 21:2616. [PubMed: 21334890]
38. Ruggles EL, Flemer S, Hondal RJ. *Biopolymers*. 2008; 90:61. [PubMed: 18008337]
39. Hrycyna CA, Yang MC, Clarke S. *Biochemistry*. 1994; 33:9806. [PubMed: 8068661]
40. Hrycyna CA, Clarke S. *Mol Cell Biol*. 1990; 10:5071. [PubMed: 2204804]
41. Choy E, Philips M. *Regulators and Effectors of Small GTPases, Pt F, Ras Family I*. 2001; 332:50.
42. Krzysiak AJ, Rawat DS, Scott SA, Pais JE, Handley M, Harrison ML, Fierke CA, Gibbs RA. *ACS Chem Biol*. 2007; 2:385. [PubMed: 17530735]
43. Perez de Castro I, Bivona TG, Philips MR, Pellicer A. *Mol Cell Biol*. 2004; 24:3485. [PubMed: 15060167]
44. Castro AF, Rebhun JF, Quilliam LA. *Methods*. 2005; 37:190. [PubMed: 16289967]
45. Winter-Vann AM, Baron RA, Wong WH, dela Cruz J, York JD, Gooden DM, Bergo MO, Young SG, Toone EJ, Casey PJ. *Proc Natl Acad Sci USA*. 2005; 102:4336. [PubMed: 15784746]
46. Tsimberidou AM, Rudek MA, Hong D, Ng CS, Blair J, Goldsweig H, Kurzrock R. *Cancer Chemother and Pharma*. 2010; 65:235.
47. Haklai R, Elad-Sfadia G, Egozi Y, Kloog Y. *Cancer Chemother and Pharm*. 2008; 61:89.
48. Jansen B, Schlagbauer-Wadl H, Kahr H, Heere-Ress E, Mayer BX, Eichler HG, Pehamberger H, Gana-Weisz M, Ben-David E, Kloog Y, Wolff K. *Proc Natl Acad Sci USA*. 1999; 96:14019. [PubMed: 10570191]
49. Judd WR, Slattum PM, Hoang KC, Bhoite L, Valppu L, Alberts G, Brown B, Roth B, Ostanin K, Huang L, Wettstein D, Richards B, Willardsen JA. *J Med Chem*. 2011; 54:5031. [PubMed: 21661760]
50. Brown MJ, Milano PD, Lever DC, Epstein WW, Poulter CD. *J Am Chem Soc*. 1991; 113:3176.
51. McCabe JB, Berthiaume LG. *Mol Biol Cell*. 1999; 10:3771. [PubMed: 10564270]
52. Castro AF, Rebhun JF, Quilliam LA. *Methods (San Diego, Calif)*. 2005; 37:190.
53. Copeland, Robert A. *Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medicinal Chemists and Pharmacologists*. Wiley Press; 2005. p. 131
54. Cer RZ, Mudunuri U, Stephens R, Lebeda FJ. *Nucl. Acids Res*. 2009; 37:W441–W445. [PubMed: 19395593]

Figure 1. Phenoxyphenyl-farnesylcysteine (**a**, POP-FC), phenoxy-phenyl-3-methylbutenyl-biphenyl farnesylcysteine (**b**, POP-3MB-FC) and anthranilic farnesylcysteine (**c**).

Figure 4. Mode of inhibition and K_I determination of compound 1a

1a displays mixed-type inhibition. Lineweaver-Burk plots illustrate the mode (mixed) of inhibition of hLcmt by **1a**. These are double reciprocal plots from AFC substrate curves with hLcmt, in the presence of the indicated concentration of inhibitor, which were obtained as described in Materials and Methods. The K_{IC} is $2.6 \pm 0.5 \mu\text{M}$ and the K_{IU} is $10.5 \pm 1.4 \mu\text{M}$.

Figure 5. Mode of inhibition and K_I determination of compound 1b

The phenoxyphenyl analog, **1a**, demonstrated mixed-type inhibition with a K_{IC} of $1.2 \pm 0.2 \mu M$ and a K_{IU} of $11.2 \pm 2.4 \mu M$. Lineweaver-Burk plots were obtained by the same method as that described for Figure 4.

Figure 6. 1b alters the subcellular localization of GFP K-Ras in Jurkat T cells

Jurkat T cells were transiently transfected with GFP-K-Ras and treated with DMSO or **1b** at indicated concentrations for 24 hrs. (A) Histograms depict the differences in GFP-K-Ras localization after indicated treatments. Differences were defined as partial or complete loss of normal localization, as defined by the images shown in (B). Results are representative of three independent experiments.

Figure 7. Inhibition of anti-CD-3 e dependent Erk phosphorylation and Ras activation in Jurkat T cells following 1b treatment (25μM)

Jurkat T cells were treated with vehicle, simvastatin (36.7 μM) or **1b** for 24 h followed by stimulation with anti-CD-3e (20μg/mL) (A) The phosphorylation of Erk was detected by immunoblotting the cell lysate with an anti-phospho-Erk antibody (top). The active form of Ras was precipitated from the cell lysate with a GST-RBD fusion protein. Precipitated Ras was detected by immunoblotting using an anti-pan-Ras antibody (bottom). Immunodetection of GAPDH was used as the loading control. Erk phosphorylation (B) and Ras activation (C) were quantified using ImageJ (NIH). The results are representative of three independent experiments.

(a) Synthesis of 2-(phenylthio)benzoic acid

(a) Potassium hydroxide, 5 eq.; Cu powder, 0.1 eq.; water (86%)

(b) Synthesis of 2-(phenoxymethyl)benzoic acid

(a) NCS, dimethylsulfide, DCM, 73% (b) NaH, DMF, 0°C to rt, 4h, 62% (c) Mg turnings, 1,2 dibromoethane, CO₂ quench, then 10% HCl, 68%.

(c) Synthesis of 2-(benzyloxy)benzoic acid

(a) NaH, DMF, 0°C to rt, 4h, 67% (b) Mg turnings, 1,2 dibromoethane, CO₂ quench, then 10% HCl 78%.

(d) Synthesis of 2-((benzyloxy)methyl)benzoic acid

(a) NaH, DMF, 0°C to rt, 4h, 77% (b) Mg turnings, 1,2 dibromoethane, CO₂ quench, then 10% HCl, 45%.**Scheme 1. Synthesis of various carboxylic acids**

Scheme 2.

General Synthetic Scheme showing Synthesis of Analogs 2a-2h

Scheme 3. Synthesis of Prenyl-Modified Phenoxy-phenyl Analogs

Scheme 4. Synthesis of N-Methyl POP-FC

Table 1

Inhibition profiles of the analogs

Structure	Analog	IC ₅₀ (μM) ^{a,b}
AFC (Negative Control)	NA
1a (POP-FC - Pos. Control)	6.2 ± 0.7 ^a
1b (POP-3MB-FC) - Pos. Control	2.5 ± 0.3 ^a
2a	63 ^b
2b	22.6 ± 1.2 ^a
2c	38 ^b
2d	14.7 ± 0.8 ^a
2e	58 ^b
2f	75 ^b
2g	13.4 ± 0.6 ^a
2h	49 ^b
17	7.8 ± 0.4 ^a
18	149 ^b
19	38 ^b

Structure	Analog	IC ₅₀ (μM) ^{a,b}
20	6.7 ± 0.4 ^a
21	210 ^b

^aIC₅₀ values are mean of three experiments, were determined using the vapor diffusion assay and calculated using GRAPH-PAD PRISM 5.0. Concentration of the substrate used (AFC) was 25 μM.

^bEstimated IC₅₀ values obtained using an in-house equation ($y = -26.813x + 77.293$; where y = percent inhibition at 10 μM and $x = \log [\text{IC}_{50}]$) that correlates percent inhibition value at 10 μM inhibitor concentration (in presence of 25 μM substrate, AFC, concentration) to experimental IC₅₀ values. This logarithmic correlation has a $R^2 = 0.85$.