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Complete List of Authors:	Butler, Kyle; Icahn School of Medicine at Mount Sinai, Structural and Chemical Biology Bohn, Kelsey; Purdue University Hrycyna, Christine; Purdue University, Chemistry Jin, Jian; Icahn School of Medicine at Mount Sinai, Department of Structural and Chemical Biology



Non-Substrate Based, Small Molecule Inhibitors of the Human Isoprenylcysteine Carboxyl Methyltransferase

Kyle V Butler^{a#}, Kelsey Bohn^{b#}, Christine A. Hrycyna^{b*}, Jian Jin^{a*}

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Activating mutations of human K-Ras proteins are among the most common oncogenic mutations, present in approximately 30% of all human cancers. Posttranslational modifications to K-Ras guide it to the plasma membrane and disruption of this localization inhibits the growth of Ras-driven cancers. The human isoprenylcysteine carboxyl methyltransferase (hlcmt) enzyme catalyzes the final ω -carboxyl methylesterification of the C-terminal farnesyl cysteine of K-Ras, which is necessary for its proper localization. Thus, hlcmt inhibition is regarded as a promising cancer therapy. A high quality inhibitor of hlcmt with *in vivo* activity would advance hlcmt research and drug development. Herein, we report the results of a screen for small molecule hlcmt inhibitors in a library of molecules that were not hlcmt substrate analogs. The lead compound identified by this screen (**1**) was modified to remove chemical liabilities and to increase potency. The most potent resulting compound (**5**) inhibited hlcmt *in vitro* with low micromolar potency ($IC_{50} = 1.5 \pm 0.2 \mu\text{M}$) and was kinetically characterized as a competitive inhibitor for prenylated substrates and a non-competitive inhibitor for the cofactor and methyl donor S-adenosylmethionine (SAM). These inhibitors offer important structure activity relationships for the future development of hlcmt inhibitors with *in vivo* activity.

Introduction

Small molecule inhibitors of K-Ras oncoproteins are perhaps the most sought after compounds in oncology drug discovery, as these proteins carry activating mutations in about 30% of human cancers.^{1, 2} Possibly due to the high affinity of K-Ras for GTP and the high concentrations of GTP in cells, direct inhibition of K-Ras has been difficult to achieve, although novel inhibitors of K-Ras^{G12C} have been described recently that target a separate binding pocket on the enzyme.³ K-Ras must be localized to the plasma membrane to properly function. Therefore, molecules that interfere with its localization could be effective inhibitors.^{4, 5} In order to be localized to the plasma membrane, newly synthesized K-Ras is modified posttranslationally by sequential prenylation, proteolytic and methylation reactions. K-Ras is first isoprenylated on the C residue of its CaaX motif (where C = cysteine, a = aliphatic residue, X = one of several amino acids, usually Ser, Met, Ala, Gln, or Leu) by farnesyltransferase (FTase

); the three C-terminal residues are proteolyzed by Ras-converting enzyme 1 (Rce1); finally, isoprenylcysteine carboxyl methyltransferase (Icmt) catalyzes transfer of a methyl group from S-adenosylmethionine (SAM) to the terminal cysteine carboxy terminus, resulting in formation of a methyl ester.⁶⁻¹⁰ Inhibiting any of these processing events disrupts Ras localization and its ability to stimulate cell growth.¹¹ Inhibitors of FTase have potent anticancer properties *in vitro*, but have failed in clinical trials due to alternative prenylation of Ras by geranylgeranyltransferases.¹ Effective inhibitors of hlcmt would be desirable therapeutics. Genetic ablation of Icmt in mice causes mislocalization of Ras and inhibits the cell proliferation and transformation caused by activating Ras mutations.^{12, 13} hlcmt inhibitors may also be useful as treatments for Hutchinson-Gilford progeria syndrome (HGPS). HGPS results from accumulation of the CaaX-containing protein prelamin A at the nuclear envelope. A hypomorphic hlcmt allele prevented appearance of the HGPS phenotype in mice carrying the progeria-causing ZMPSTE24 deficiency.¹⁴

Multiple hlcmt inhibitor chemotypes have been reported, most of which are either substrates or substrate analogs.¹⁵⁻²⁰ There are few reports of hlcmt inhibitors that are not based on farnesylated substrates, including members of the tetrahydropyranyl series and cysmethynil and its analogs.²¹⁻²⁶ The tetrahydropyranyl compounds were shown to have low nanomolar potency *in vitro*, but lacked robust antiproliferative activity.²¹ Cysmethynil, which is the most well-characterized hlcmt inhibitor, has an *in vitro* IC_{50} of 1.5 μM and causes Ras mislocalization in cells at micromolar concentrations.^{22, 23} These molecules are currently being used successfully as tools

^a Departments of Structural and Chemical Biology, Oncological Sciences, and Pharmacology and Systems Therapeutics, Icahn School of Medicine at Mount Sinai, New York, New York 10029, United States.

^b Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, Indiana 47907, United States.

These authors contributed equally to this work, * Corresponding author, email: jian_jin@mssm.edu or hrycyna@purdue.edu. † The authors declare no competing interests.

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to interrogate pathways and the cellular role of isoprenylcysteine methylation but not as therapeutics.²⁷ Unfortunately, thus far, inhibitors of *hlcmt* with both high potency and robust cellular activity have not been identified. Thus, there is an urgent need for *hlcmt* inhibitors with potent *in vivo* activities for cancer and progeroid disorder drug discovery. To meet this need, we developed a series of non-substrate *hlcmt* inhibitors featuring a novel chemotype. The most potent compound in this series has an *in vitro* IC₅₀ value of 1.5 ± 0.2 μM, shows competitive inhibitory kinetics for a farnesylated substrate and non-competitive kinetics for the cofactor SAM.

Results

Screening

To identify novel *hlcmt* inhibitors, we screened a library of 700 compounds in an *in vitro* vapor diffusion assay developed for *hlcmt* activity. Crude membrane extracts expressing human *hlcmt* (*hlcmt*) were prepared as described.¹⁵ The library was composed of commercially available compounds and also compounds synthesized as part of ongoing methyltransferase inhibitor drug discovery projects, including the soluble histone methyltransferases G9a and SETD8.^{28–34} We used the vapor diffusion assay for *hlcmt* activity for both the primary screen of the inhibitors and for subsequent IC₅₀ determination.^{7, 15} For the screen, compounds were assayed at a single concentration of 10 μM. Any compound that reduced activity to less than 15% of the wild-type control was considered for further screening. Confirmed hits were subjected to an IC₅₀ determination assay. The most potent of this group was compound **1**, which demonstrated an IC₅₀ value of 14 μM (Figure 1). Compound **1** was synthesized by the Petasis multicomponent reaction, and compounds of this type are frequently found in commercially available HTS collections. We had several initial concerns with **1** as a lead compound. Phenolic Mannich-bases like **1** are known to be pan-assay interference compounds and are sometimes false leads for drug discovery.³⁵ Also, the compound can readily decompose in acidic solution through the retro-Mannich pathway. We modified the structure in order to nullify these liabilities as described below (Figure 1).

Medicinal Chemistry

The C-N bond of **1** is labile, and we removed this liability by removing the nitrogen. Additionally, we replaced the problematic hydroxyquinoline chemophore with hydroxynaphthalene. Synthesis was accomplished by sequential nucleophilic attack on a Weinreb amide, followed by dehydroxylation of the resulting tertiary alcohol. The resulting compound, **2**, had an *in vitro* IC₅₀ of 18 μM, similar to the lead. Saturation of the C-C bond linking the two arenes and the tetrahydropyran, as in the alkene-containing analog, **3**, increased the potency to an IC₅₀ of 7.7 μM. Replacing the hydroxynaphthalene with naphthalene resulted in a further

gain in potency. The tertiary alcohol compound **4** has an IC₅₀ of 2.2 μM and the alkene **5** (Figure 2) has an IC₅₀ of 1.5 μM. Thus, the problematic lead compound, **1**, was transformed into a compound lacking the original liabilities, with a ~9-fold increase in potency.

We next attempted to modify the thiophene portion of the molecule. Replacement of thiophene with phenyl gave the inactive compound, **6**. Removal of the thiophene, as in compounds **7** and **8**, also abolished activity. We also attempted to further minimize the lead by replacing naphthalene with a phenyl group, giving compounds **9** and **10**. However, both were inactive. The most potent compound, **5**, has a cLogP value of 4.9 and is very planar. Both of these factors will decrease compound solubility, and consequently, may compromise *in vivo* potency. We sought to improve solubility by replacing the ether oxygen with a protonatable nitrogen and by saturating the alkene. The resulting compounds, **11** and **12**, however, were not active. The non-substrate analog inhibitors were not as potent as some previously reported substrate analogs like FTPA Triazole 10n.¹⁵

In order to determine the mode of inhibition by **5**, our most potent compound, detailed kinetic assays were performed using crude yeast membrane preparations expressing *hlcmt*. Lineweaver-Burk analyses demonstrated that **5** is a competitive inhibitor with the *hlcmt* minimal substrate, N-acetyl-S-farnesyl cysteine (AFC) (K_i = 1.1 ± 0.2 μM) and a non-competitive inhibitor with SAM (K_i = 2.9 ± 0.2 μM) (Figure 3).

Cell killing assays were conducted to assess the ability of **5** to inhibit the growth of MIA-PaCa2, a pancreatic cancer cell line expressing a G12C K-Ras mutation. Compound **5** MIAPaCa cells with an IC₅₀ of 31.9 ± 2.3 μM. Compound **5** killed Panc-1 cells (G12D mutant K-Ras) with an IC₅₀ of 78 μM.

Discussion

Screening of a library of 700 unique compounds identified the lead, **1**, as an inhibitor of *hlcmt* with an IC₅₀ of 14 μM in an *in vitro* methyltransferase assay. The structure of **1** was modified to replace the labile C-N bond and to replace the phenolic Mannich base functional group associated with assay interference. This process led to the most potent compound in this series, **5**, with an IC₅₀ value of 1.5 μM (Figures 1 and 2). The *in vitro* IC₅₀ value of **5** is similar to the reported for the well-characterized *hlcmt* inhibitor, cysmethynil.²³ All but two of the hydrogen-bond forming atoms were removed in the transformation of the original lead to **5**, leaving a planar, hydrophobic compound. Efforts to decrease the cLogP of **5** or to add solubility-enhancing groups were unsuccessful. Compound **5** is much more simple than the parent **1**, and has only two hydrogen bond acceptors. The high ratio of C/H atoms relative to heteroatoms in **5** suggests that its affinity for *hlcmt* is largely entropy-driven. It is likely that functional groups could be added to **5** that would form hydrogen bonds with *hlcmt*, improving the enthalpy component of the binding

Cmpd.	IC ₅₀ (μM)	Structure	Cmpd.	IC ₅₀ (μM)	Structure
1	14 ± 1		7	>50	
2	18 ± 5		8	>50	
3	7.7 ± 0.5		9	>50	
4	2.2 ± 0.2		10	>50	
5	1.5 ± 0.2		11	>50	
6	>50		12	>50	
			FTP triazole 10n	0.4 ± 0.1 ^a	

Figure 1: Structures and IC₅₀ values of compounds. Compounds containing a stereocenter were prepared as the racemate. Assays were conducted three separate times in duplicate. a.) The FTPA triazole is shown for comparison.

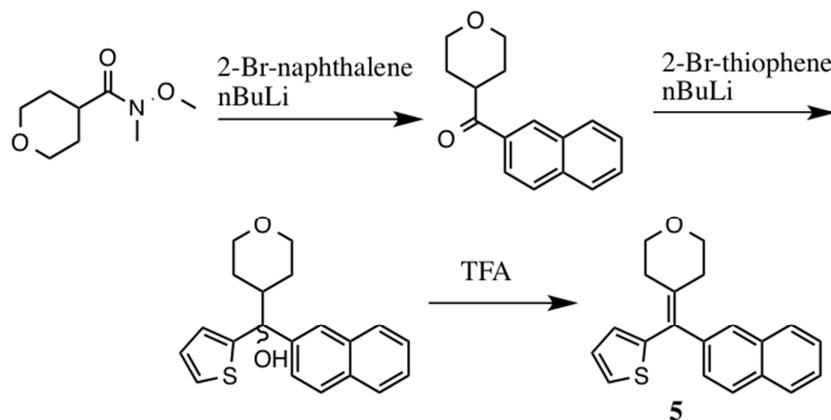


Figure 2: Synthesis of **5**. Reagents and conditions: a.) 2-Br-naphthalene, n-Butyllithium; b.) 2-Br-thiophene, n-Butyllithium; c.) Trifluoroacetic acid.

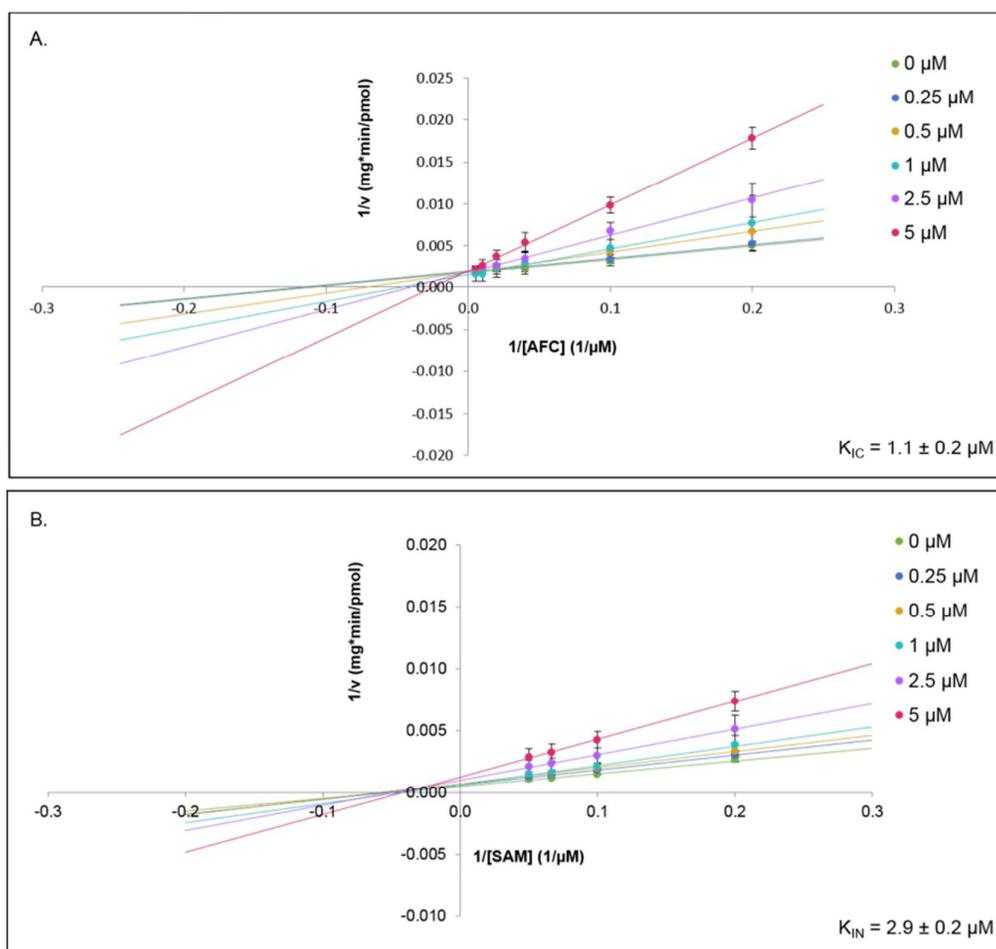


Figure 3: Compound **5** is competitive with AFC and non-competitive with SAM. (A) Specific activity of ICMT was determined with increasing concentrations of AFC for varying concentrations of **5** while keeping SAM concentration constant at 5 μM . (B) Specific activity of hIcmt was determined with increasing concentrations of [^{14}C]-SAM for varying concentrations of **5** while keeping AFC concentration constant at 25 μM .

affinity, but in the absence of a co-crystal structure, rationally introducing such groups would be difficult. The crystal structure of an hlcmt ortholog has been solved, however homology only exists to the C-terminal half of the enzyme and does not include the prenylated substrate binding site.³⁶

The lead compound **1**, contains a known pan-assay interference (PAINS) functional group. PAINS interfere nonspecifically in multiple, unrelated biochemical assays. Optimization of these compounds by medicinal chemistry frequently wastes resources. However, the PAINS functional group of **1** was removed, yielding a structurally similar but more potent inhibitor. This result demonstrates that PAINS structures can serve as viable leads for drug discovery, as has been noted by Baell et al.³⁷

A Lineweaver-Burk plot revealed **5** to be competitive with the prenylated peptide substrate, AFC and non-competitive with SAM. The substrate-competitive enzyme inhibition of lcms is inherently druggable, as the enzyme must present a large nonpolar surface to bind the farnesyl group, concomitantly presenting a binding site for lipophilic small molecules.

Conclusions

Potent and effective inhibitors of hlcmt could be useful treatments for K-Ras-driven malignancies. No clinically effective inhibitors of K-Ras function exist, so these treatments are urgently needed. The inhibitors described in this work could serve as useful leads for drug development towards the discovery of highly effective hlcmt inhibitors. We have significantly advanced the potency of this series of compounds with a few functional group substitutions. We anticipate that further optimization of this series will result in high potency inhibitors.

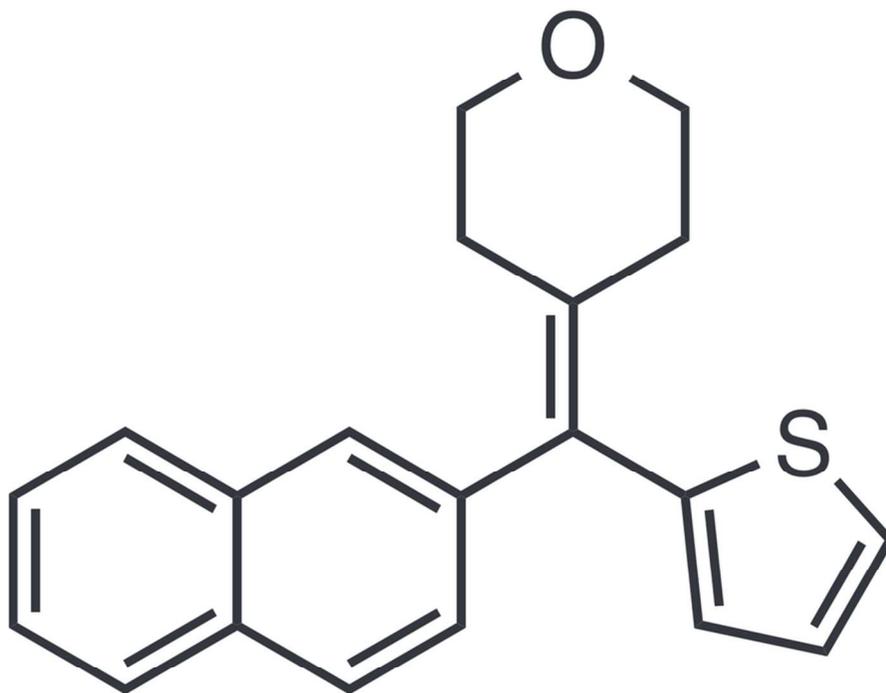
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hICMT IC₅₀: 1.5 μ M
Substrate Competitive

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