

S-Farnesyl-Thiopropionic Acid Triazoles as Potent Inhibitors of Isoprenylcysteine Carboxyl Methyltransferase

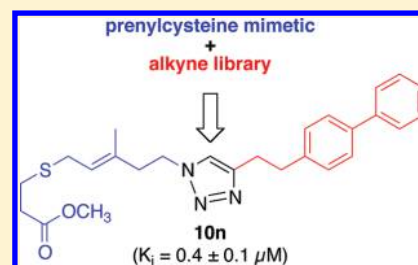
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S Supporting Information

ABSTRACT: We report the design and synthesis of novel FTPA-triazole compounds as potent inhibitors of isoprenylcysteine carboxyl methyltransferase (Icmt), through a focus on thioether and isoprenoid mimetics. These mimetics were coupled utilizing a copper-assisted cycloaddition to assemble the potential inhibitors. Using the resulting triazole from the coupling as an isoprenyl mimetic resulted in the biphenyl-substituted FTPA triazole **10n**. This lipid-modified analogue is a potent inhibitor of Icmt ($IC_{50} = 0.8 \pm 0.1 \mu M$; calculated $K_i = 0.4 \mu M$).

KEYWORDS: Isoprenylcysteine carboxyl methyltransferase (Icmt), Ras, prenylcysteine, dipolar cycloaddition, S-farnesyl-thiopropionic acid (FTPA), triazole



The post-translational processing of members of the Ras protein superfamily is under active investigation because approximately 20% of human cancers result from mutated Ras proteins.¹ These proteins contain a -CaaX motif that is first isoprenylated on the cysteine thiol with a farnesyl group by farnesyltransferase (FTase) or a geranylgeranyl group by geranylgeranyltransferase-1 (GGTase-1). Following lipidation, two critical modifications occur sequentially at the endoplasmic reticulum (ER) by membrane-associated enzymes. First, the endoprotease Ras converting enzyme-1 (Rce-1) cleaves the terminal -aaX residues, resulting in a newly exposed prenylcysteine at the C terminus of the protein. Second, isoprenylcysteine carboxylmethyltransferase (Icmt) methyl esterifies the carboxylate using S-adenosyl-methionine (SAM) as the methyl donor. The increased hydrophobicity imparted by these modifications is thought to allow for proper localization of the Ras proteins to the plasma membrane and thus their function.²

Because blocking Ras localization should prevent its functioning in tumor progression, this post-translational modification pathway has been the target for the development of therapeutics against mutant Ras-based tumors. To this end, potent FTase inhibitors were developed that showed promise against some tumors but proved ineffective against K-Ras driven tumors,³ due in part to alternative geranylgeranylation of K-Ras. We chose to focus on developing inhibitors of Icmt, the only known enzyme that catalyzes the methyl esterification of the terminal prenylcysteine.⁴ Genetic knockout studies have provided support for the importance of Icmt-mediated methylation in Ras signaling.⁵

Substrate specificity studies revealed several key elements necessary for recognition by Icmt, including a C₁₅ or C₂₀ isoprenyl lipid and a requirement for a thioether bridge between the lipid and the carboxylate motifs.^{6,7} Early work by

Rando et al. identified the minimal substrate for Icmt as farnesyl thiopropionic acid (FTPA, **1**).⁸ Using this information as a starting point, inhibitors of Icmt were developed from studies of rotationally restricted prenylcysteines,⁹ library screening efforts,¹⁰ and amide-^{11,12} and isoprenyl-modified¹³ prenylcysteines. In recent studies, we have established the prenylcysteine analogue POP-3MB as a low micromolar inhibitor of human Icmt.¹²

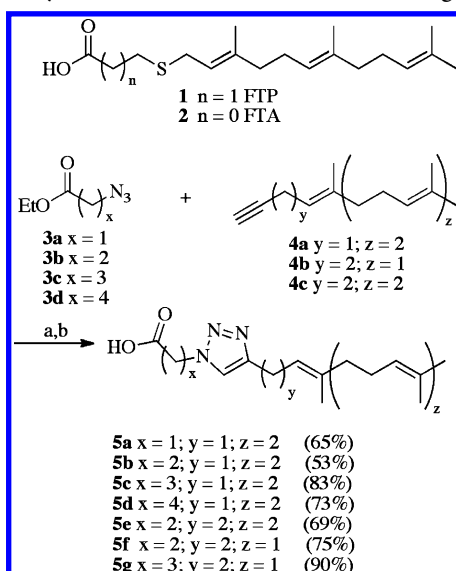
We have now utilized FTPA as a starting point for nonamino acid Icmt inhibitor scaffolds, incorporating a triazole as a thioether or isoprene mimetic. This approach allowed us to utilize the synthetic advantages of copper-assisted dipolar cycloaddition (click chemistry) for rapid and facile analogue assembly.¹⁴ Herein, we report the synthesis and evaluation of FTPA analogues assembled utilizing click chemistry. Triazole **10n** represents our most potent Icmt inhibitor to date, with an in vitro IC_{50} of $0.8 \pm 0.1 \mu M$.

In the initial design, a 1,4-disubstituted 1,2,3-triazole was positioned as the thioether replacement and cysteine backbone modifier that would join the lipid and carboxylate motifs of the FTPA analogues (Scheme 1). This approach had two key advantages: (a) It replaced the potentially labile allylic thioether, and (b) the building blocks needed for the reaction were readily available. Azido-esters **3a–d** underwent cycloaddition with alkynes **4a–c**,^{15,16} followed by saponification to afford **5a–g** in acceptable yields. This synthetic sequence generated analogues with varying flexibility between the carboxylate, the triazole, and the lipid motifs.

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Scheme 1. Synthesis of Triazole for Sulfur Analogues^a

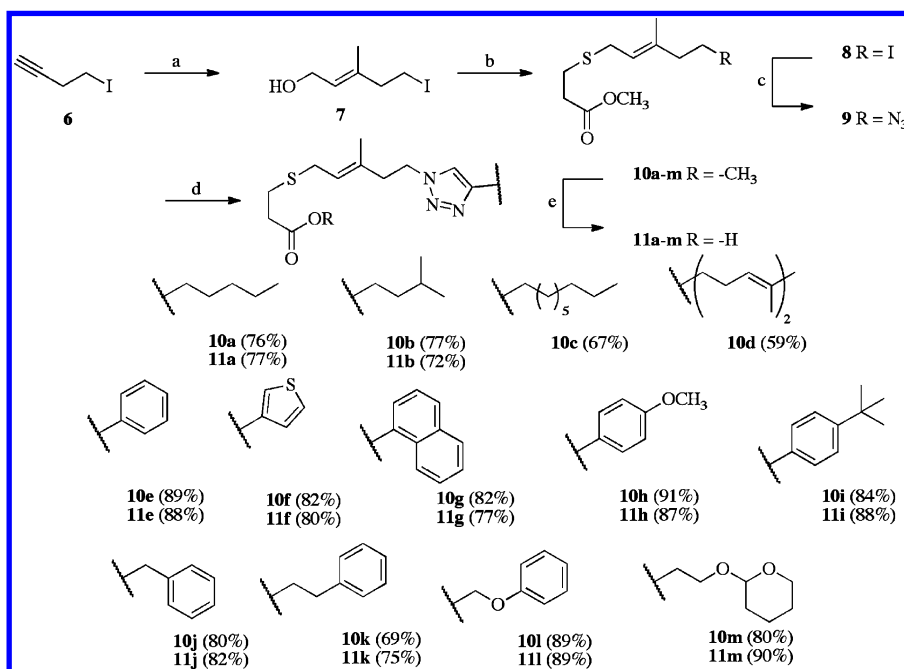
^aReagents and conditions: (a) Sodium ascorbate, cupric sulfate, *t*BuOH/H₂O, room temperature. (b) LiOH, MeOH; 20–80% over two steps. The number in parentheses indicates Icmt specific activity as percent of AFC control in the presence of 10 μ M inhibitor.

These analogues (Scheme 1) were evaluated for activity using a vapor diffusion assay.¹⁷ Compounds **5a–g** were modest inhibitors of Icmt but were not substrates. Structure–activity relationships (SAR) determined an optimal two-methylene bridge (**5b**) between the carboxylate and the triazole. Triazole-containing analogues based on the FTPA structural motif thus warranted further exploration, as this approach could generate diverse compounds quickly and easily.

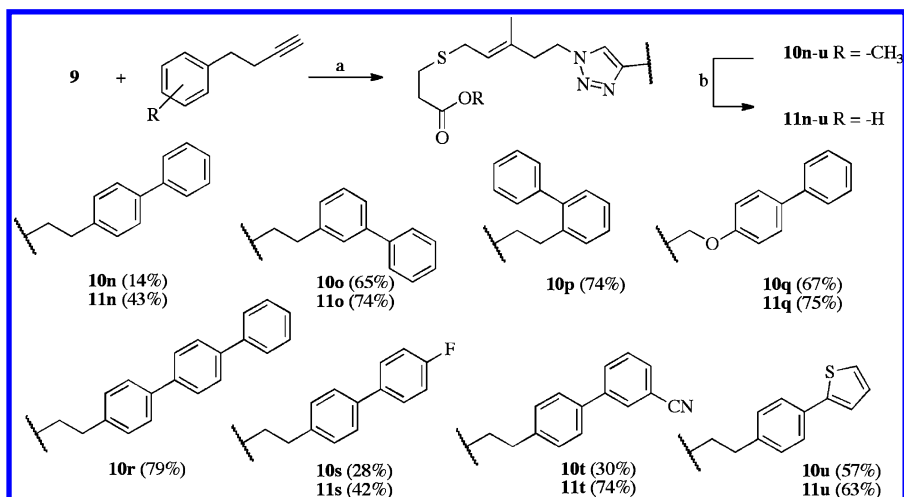
Previous efforts examined isoprenyl-modified AFC analogues as Icmt inhibitors.¹³ These efforts demonstrated that the correct (*E*)-geometry of the first isoprene must be maintained as well as inclusion of the sulfur heteroatom.⁷ Furthermore, saturated lipids were also not mimetics,¹³ suggesting that specific “druglike” lipid structural motifs will interact with the active site of Icmt. We also took compounds developed previously as ligands for other isoprenyl binding pockets into consideration when designing our analogues. Aryl mimetics of isoprenoids have been utilized in the development of nanomolar squalene synthase¹⁸ and FTase ligands,^{19–21} and thus, we focused on this approach in our subsequent set of potential Icmt inhibitors.

The synthesis of the FTPA-triazoles began from iodide **6**, which underwent Zr-assisted carboalumination,²² affording the desired trisubstituted olefin **7** (Scheme 2). Corey–Kim chlorination²³ and alkylation with methyl 3-mercaptopropionate resulted in ester **8**. Sodium azide displaced the primary iodide, affording the desired cycloaddition partner **9**. Copper-mediated cycloaddition with a variety of terminal alkynes (with a focus on aromatic moieties) provided the FTPA-triazole analogues with good yields. These compounds were evaluated as both methyl esters (**10a–m**) and carboxylates (**11a–m**) following saponification.

These FTPA-triazole analogues were evaluated using the vapor diffusion assay. Neither the methyl esters **10a–m** nor the carboxylates **11a–m** were Icmt substrates. The alkyl analogues **10a–d** and **11a–d** were modest inhibitors, and the esters showed inhibitory activity comparable to the acids. However, aryl rings attached to the triazole (**10e–i** and **11e–i**) resulted in both ester and acid compounds that were poor inhibitors of Icmt. These data suggest that neither saturated lipids nor rigid aryl groups directly joined to the triazole satisfied the structural requirements necessary for inhibition.

Scheme 2. Synthesis of Triazole Substitutions in FTPA^a

^aReagents and conditions: (a) Me₃Al, Cp₂ZrCl₂, (HCO)_n, 0 °C–room temperature, 60%. (b) (i) *N*-Chlorosuccinimide, Me₂S, DCM, –40 °C–room temperature; (ii) methyl thiopropionate, DIEA, DCM, 0 °C–room temperature, 55% over two steps. (c) NaN₃, DMF, 80 °C, 78%. (d) Sodium ascorbate, cupric sulfate, *t*BuOH/H₂O, room temperature, 35–97%. (e) LiOH, MeOH, room temperature, 40–90%. The number in parentheses indicates Icmt specific activity as percent of AFC control in the presence of 10 μ M inhibitor.

Scheme 3. Triazole Substitutions in FTPA: Use of Biphenyl Alkynes^a

^aReagents and conditions: (a) Sodium ascorbate, cupric sulfate, *t*BuOH/H₂O, room temperature; 36–78%. (b) LiOH, MeOH, room temperature, 50–96%. The number in parentheses indicates Icmt specific activity as percent AFC control with 10 μ M inhibitor.

Natural isoprenylcysteine substrates have a high degree of flexibility, and the aryl analogues described above possessed an inflexible motif. Increased inhibition of Icmt was afforded with increased spacing between the triazole and the aryl motifs. Analogue **10k** was the most potent analogue in this series, with an IC₅₀ of 41 \pm 5 μ M. Alternatively, **11m**, with both a polar and a nonaromatic tail, displayed poor Icmt inhibition (IC₅₀ > 100 μ M). These data suggest that incorporation of aryl groups within the isoprenyl region via a triazole must be accompanied by a concomitant introduction of flexibility.

Because Icmt can also efficiently methylate geranylgeranylated substrates,⁶ we hypothesized that further extension into the region of the terminal isoprene could be utilized for enhanced binding of our FTPA-triazole analogues. To examine this hypothesis, a series of biphenyl alkynes were utilized, as we have previously shown that the biphenyl group is an effective isoprene mimetic.^{12,13,19} These analogues also included incorporation of increased spacing between the triazole and the biphenyl motifs to afford flexibility. Of these analogues, the most potent inhibitor of Icmt in vitro was **10n**. In our single point assay, **10n** (10 μ M) decreased the specific activity of Icmt to 14% of the no inhibitor control (Scheme 3). We experimentally determined an in vitro IC₅₀ of 0.8 \pm 0.1 μ M for **10n** and a calculated K_i of 0.4 μ M (Cheng and Prusoff method²⁴). Further biochemical evaluation found **10n** to be a competitive inhibitor. Because the methyl ester **10n** was more potent than the corresponding carboxylate, **11n**, modification of the carboxylate group may represent a new approach for inhibitor design.²⁵

One particularly attractive feature of **10n** is its ease of synthesis; it was prepared in five linear steps from homopropargyl iodide **6** in an unoptimized 14% overall yield. This route facilitated rapid additional SAR efforts through the synthesis of modified biphenyl analogues. Analogues with alterations to the orientation of the biphenyl moiety (**10o–p**) decreased Icmt inhibition. The ester **10o** was nearly 30 times less potent than **10n**, with an IC₅₀ of 29 \pm 5 μ M (Table 1), and the *ortho*-**10p** analogue possessed further reduced inhibitory activity, indicating the importance of the *para*-biphenyl motif. Furthermore, inclusion of an oxygen linker (**10q**) was also detrimental to inhibition. Compounds containing electron-withdrawing groups

in the terminal aromatic ring (**10s**, **10t**) were approximately twice as potent as the electron rich analogue **10u**.

Table 1. Cellular Activities (IC₅₀) of Select FTPA-Triazole Analogues

compd	cell-free (μ M)	MEF		PaTu-8902 (μ M) ^{b,c}
		Icmt ^{-/-} (μ M) ^{a,c}	Icmt ^{+/+} (μ M) ^{a,c}	
10g	ND	>100	96 \pm 0.01	ND
10n	0.8 \pm 0.1	>100	33 \pm 1	8 \pm 3
10o	29 \pm 5	>100	55 \pm 2	ND
FTS	25.5 \pm 2	14.3 \pm 2.2	15.3 \pm 1.8	7.8 \pm 1.7

^aMEFs are plated in 96-well plates at a density of 1000 cell/well in DMEM supplemented with 10% FBS. After incubation for 24 h, the media are replaced with DMEM supplemented with 5% FBS and **10n** (various concentrations) or DMSO (0.1%). The cell viability is determined after 5 days using MTT. Twenty microliters of MTT (5 mg/mL) is added to each well and incubated for 4 h at 37 $^{\circ}$ C. Afterward, the media are removed, and 150 μ L of DMSO are added to each well. The absorbance is determined at 590 nm using a Molecular Devices VERSAmix microplate reader. ^bIC₅₀ determinations were performed in triplicate and calculated with Graphpad Prism V4. ^cND, not determined.

The lead compound (**10n**) was tested for efficacy in vitro in several cell line model systems. First, MEF cells derived from Icmt^{+/+} and Icmt^{-/-} animals⁴ were treated with increasing concentrations of **10n** for 24 h, and cell viability was measured using a standard MTT assay (Table 1). In MEF Icmt^{-/-} cells, **10n** demonstrated an IC₅₀ of >100 μ M, whereas an IC₅₀ of 33 μ M was observed for wild-type MEF Icmt^{+/+} cells. Two analogues that are structurally related to **10n** but are not Icmt inhibitors in vitro were also examined in this assay. Analogues **10g** and **10o** had IC₅₀ values in MEF Icmt^{+/+} cells of 96 and 55 μ M, respectively, and did not affect viability in MEF Icmt^{-/-} cells (Table 1). Furthermore, treatment with FTS (*S*-farnesylthiosialicylic acid), an Icmt inhibitor that also possesses other cellular effects²⁷ and has clinical potential,²⁸ showed equivalent activity in both cell lines. Finally, as activating point mutations in K-Ras can be found in approximately 90% of pancreatic cancers,²⁹ the in vitro efficacy of **10n** was examined in the highly metastatic and “K-Ras

addicted³⁰ pancreatic cancer cell line PaTu-8902. An IC_{50} of 8 μ M was determined for **10n**, which is comparable to the known Icm1 inhibitor FTS (Table 1).

Proper posttranslational processing is a prerequisite for Ras membrane localization and function. To determine the cellular effects of our most potent inhibitor **10n**, the membrane localization of fluorescently tagged K-Ras was visualized in the presence and absence of **10n**.^{26–31} Jurkat T-cells that were transfected with GFP-K-Ras and treated with either delivery vehicle alone, the statin drug simvastatin (which blocks K-Ras prenylation), or **10n** were fixed, and GFP-K-Ras localization was visualized using fluorescent microscopy. After 24 h of treatment, GFP-K-Ras localization was categorized into three groups: normal plasma membrane localization, partial mislocalization, and complete mislocalization. This classification was determined via visual inspection of randomly selected fields containing 100 cells. The results demonstrated that **10n** led to a decrease in normal membrane localization (Figure 1). These

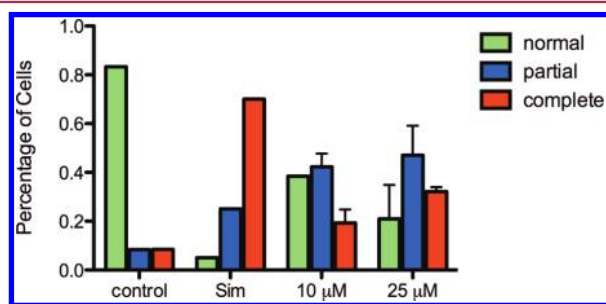


Figure 1. Mislocalization of GFP-KRas construct upon **10n** administration. Jurkat T cells (E 6.1) were treated with vehicle, 25 μ M Simvastatin (Sim), or **10n** at indicated concentrations for 24 h. Cells were overlaid onto poly-L-lysine-coated coverslips (100 μ g/mL), followed by fixation with 3.7% formaldehyde solution for 10 min. Subcellular localization of GFP-KRas was quantified using fluorescence microscopy (Olympus BH-2RFCA).

data suggest that **10n** is taken up into mammalian cells and functions to prevent K-Ras membrane localization.

FTPA-triazole **10n** was generated by removing the amide moiety present in other isoprenoid mimetics and by adding a biphenyl isoprenyl mimetic. Compound **10n** was found to be a potent inhibitor of Icm1 capable of inducing K-Ras mislocalization in a cellular model system. Furthermore, **10n** appears to be selectively cytotoxic for Icm1^{+/+} MEF cells and has low micromolar activity against a metastatic pancreatic cancer cell line. Together, these data suggest, but do not prove, that **10n** is targeting Icm1 in cells. The lead triazole analogue **10n** not only possesses higher potency than our best previous Icm1 inhibitor (POP-3MB: IC_{50} = 2.5 μ M),¹² but it also possesses a lower molecular weight (450 vs 606), a lower CLog P (5.5 vs 8.8), and is much more easily assembled. To the best of our knowledge, **10n** represents the first substrate-based compound to exhibit submicromolar inhibition against Icm1. Furthermore, **10n** exhibits significantly improved ligand efficiency³² and is thus a superior starting point for drug development efforts.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures, full experimental details, and details of biological assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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