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Amide-substituted farnesylcysteine analogs as inhibitors of human isoprenylcysteine carboxyl methyltransferase

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Abstract—*N*-Acetyl-*S*-farnesyl-L-cysteine (AFC) is the minimal substrate for the enzyme isoprenylcysteine carboxyl methyltransferase (Icmt). A series of amide-modified farnesylcysteine analogs were synthesized and screened against human Icmt. From a 23-membered library of compounds, six inhibitors were identified and evaluated further. The adamantyl derivative **7c** was the most potent inhibitor with an IC_{50} of 12.4 μM.

Numerous proteins are initially synthesized with a C-terminal –CaaX box motif, where -C is cysteine, -aa are generally two aliphatic residues, and –X is typically S, M, F, Q, or L. This motif labels the protein for a series of sequential post-translational modifications (Fig. 1). First, either a 15-carbon farnesyl or 20-carbon geranylgeranyl group is added via a thioether linkage to the cysteine by one of two soluble isoprenyltransferases (protein-farnesyltransferase, FTase, or protein-geranylgeranyltransferase I, GGTase I). Following the attachment of the prenyl group, the three –aaX residues are cleaved by the endoprotease Ras-converting enzyme 1 (Rce1), and subsequently the newly exposed cysteine carboxyl group is methylated by isoprenylcysteine carboxyl methyltransferase (Icmt). It is estimated that at least 120 mammalian proteins undergo this sequential three-step post-translational modification sequence,[1] the sum of which typically results in increased hydrophobicity and enhanced membrane association of an initially cytosolic protein.

This post-translational pathway became the subject of intense scrutiny as a target for cancer therapies, as it was determined that the oncogenic Ras family of GTPases must be farnesylated in order to properly function. Importantly, mutations in this family of proteins are responsible for approximately 20–30% of all human cancers and 90% of pancreatic cancers. A number of farnesyltransferase inhibitors (FTIs) are currently undergoing evaluation in clinical trials.[2,3] However, these agents have not exhibited significant activity in most patients with Ras-driven tumors[4] due to alternative geranylgeranylation of Ras in FTI-treated cells.[5,6]

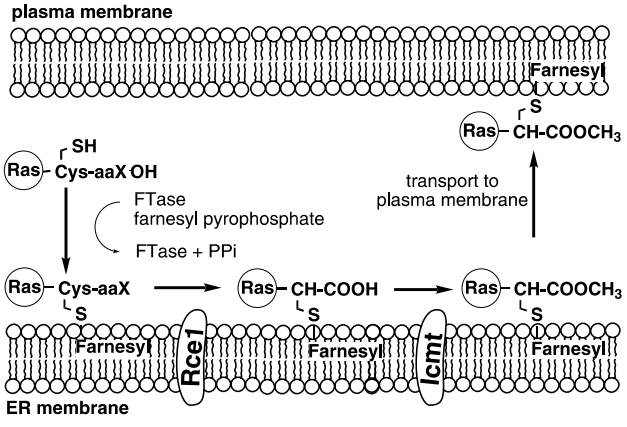

Figure 1. Post-translational modification of Ras by FTase, Rce1, and Icmt.

Keywords: Farnesylcysteine analogs; Isoprenylcysteine carboxyl methyltransferase; Post-translational modification.
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Rce1 and Icmt have been recognized for a number of years as potential alternative anticancer targets to FTase.[7] Although originally thought to be too important for cell viability due to the embryonic lethal phenotype of Rce1$^{-/-}$ and Icmt$^{-/-}$ mice,[8] a recent publication by Michaelson et al. showed that proteolysis and methylation are not as important for the proper functioning and localization of geranylgeranylated proteins as for farnesylated proteins.[9] Thus, inhibiting Rce1 or Icmt may result in a phenotype similar to that observed when inhibiting FTase alone and not like the profound toxic effects seen with dual FTase/GGTase I inhibitors.[10] We have therefore initiated an effort to generate Icmt inhibitors based on the structure of the minimal Icmt substrate *N*-acetyl-*S*-farnesyl-L-cysteine (AFC, Fig. 2, **1**) in hopes of developing potent anticancer agents as well as molecular tools to study the structure and mechanism of Icmt.

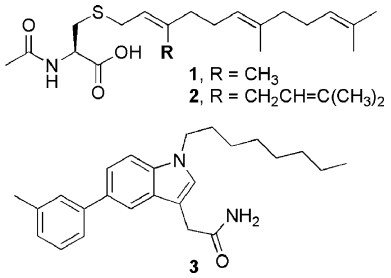


Figure 2. Structures of *N*-acetyl farnesylcysteine (AFC) (**1**), *N*-acetyl-3-isobutenylfarnesylcysteine (**2**), and cysmethynil (**3**).

Recently reported work from our laboratories has shown that selective changes in the farnesyl group of AFC can afford effective inhibitors of yeast Icmt (Fig. 2, **2**; $K_I = 17$ μM).[11] However, compound **2** was not an effective inhibitor of human Icmt. Winter-Vann et al. have also identified a novel indole-based small molecule inhibitor of human Icmt from a library screen. This compound, termed cysmethynil (**3** Fig. 2; $IC_{50} = 2.4$ μM in vitro), resulted in an Icmt-dependent Ras mislocalization, as well as a decrease in cell proliferation and anchorage-independent growth in soft agar assays.[12] In response to these promising initial reports, we have expanded our efforts toward the development of AFC-based Icmt inhibitors, and have now synthesized a series of amide-modified farnesyl cysteine (FC) analogs (Fig. 3).

The biochemical and cellular effects of other FC analogs have been previously reported. Farnesyl thiosalicylic acid (FTS) has been shown to inhibit the growth of H-Ras-driven Rat1 cells, though it is believed this effect is not solely due to inhibition of Icmt.[13] Perez-Sala et al. showed that another AFC-based compound, *S*-farnesylthioacetic acid (FTA), induced apoptosis in HL-60 cells. However, Ras was not mislocalized away from the cell membrane, suggesting a non-Icmt-specific effect of this compound.[14] Note that FTS and FTA were reported to be methyltransferase inhibitors ($IC_{50} = 2.8$ and

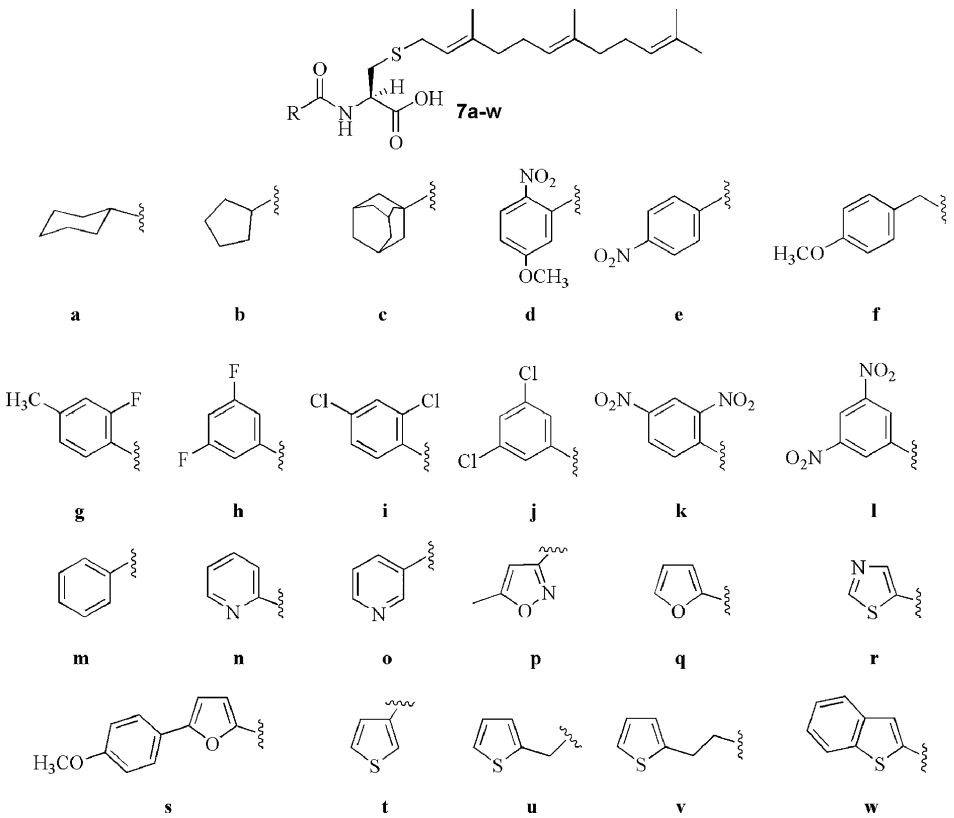
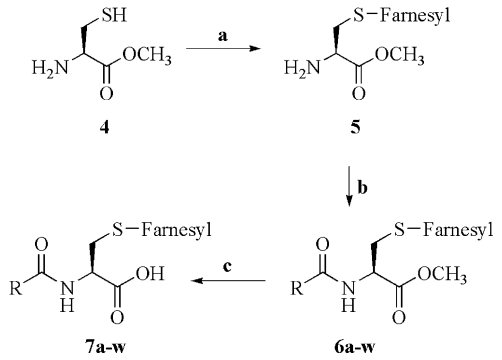


Figure 3. Structures of compounds screened against Icmt in SPA.

4.6 μM, respectively),[13,14] but these studies were performed with crude membrane preparations from tissues rather than the well-characterized recombinant human Icmt[11] that is now available. In an attempt to distinguish Icmt inhibition from other non-specific effects, Ma et al. synthesized a number of amide-modified AFC analogs.[15,16] Bulky benzoyl and pivaloyl analogs did not act as substrates, but the introduction of a flexible glycine spacer between the benzoyl group and farnesylcysteine restored substrate activity.[16] We propose that a further exploration of the structure–activity relationship for AFC analogs could lead to more potent Icmt inhibitors. It is our hypothesis that an FC-based inhibitor that has a higher affinity for the enzyme but is not a substrate will reduce the pleotropic effects associated with FTS and FTA.

Using the short synthetic route outlined in Scheme 1, a library of 23 amide-modified farnesylcysteine analogs was synthesized. Farnesylcysteine methyl ester **5** was prepared via the method of Poulter and co-workers.[17] Standard peptide coupling conditions were used for the introduction of the various carboxylic acids. HOBt, EDC, and R–COOH were dissolved in DMF, followed by the addition of *i*-Pr_2EtN. In some instances this solution required heating to dissolve the carboxylic acid prior to addition of **5**. After 2–3 h at room temperature, work-up and purification gave **6a**–**w** in 55–95% yield. Hydrolysis of the methyl ester was accomplished by dissolving **6a**–**w** in 95% ethanol at zero degrees and adding NaOH. Reactions were typically complete in one hour which after work-up and purification gave **7a**–**w** in 40–70% yield.

The synthesized compounds shown in Figure 3 were screened for Icmt inhibitory activity using a high-throughput scintillation proximity assay (SPA).[18] In the SPA, crude membranes from *Saccharomyces cerevisiae* cells overexpressing active human Icmt were incubated with 20 μM of the methyl donor *S*-adenosyl-[^{14}C-methyl]methionine (SAM) and 25 μM of the substrate *N*-biotinyl-(6-aminohexanoic)-*S*-farnesyl-L-cysteine (BFC).[19] The extent of the methylation by hIcmt is quantified by the binding of the methylated BFC to streptavidin SPA beads, and measuring the counts per minute (cpm).



Scheme 1. Reagents and conditions: (a) farnesyl-Cl, 7 N NH_3/MeOH, 0 °C to rt, 82%; (b) R–COOH, HOBt, EDC, DIEA, DMF, rt, 50–95%; (c) NaOH, 95% EtOH/H_2O, rt, 40–75%.

Table 1. Inhibition of human Icmt by selected compounds

Compound	% of control[a,c]	Enzyme inhibition IC_{50}[a,d] (μM)
None	100.0 (±19.0)	NA[b]
7c	12.8 (±7.9)	12.4 (±1.0)
7e	50.1 (±3.5)	16.6 (±1.3)
7j	33.5 (±4.3)	23.0 (±1.9)
7l	37.1 (±4.2)	14.3 (±0.8)
7s	35.1 (±2.2)	17.4 (±1.6)
7w	28.0 (±2.9)	20.3 (±2.6)

[a] Values are means of three experiments, SEM is given in parentheses.
[b] NA, not applicable.
[c] Determined using an SPA procedure.[18b]
[d] IC_{50} values determined using vapor diffusion assay[20] and calculated using GraphPad Prism 4.0.

From our initial screen of twenty-three compounds, six agents (**7c**, **7e**, **7j**, **7l**, **7s**, and **7w**) led to a 50% or greater decrease in BFC methylation, our criteria for advancing a compound into additional testing (Fig. 3). In this study, we found that FC analogs containing larger R-groups inhibited Icmt more effectively than those with smaller R-groups. In particular, the adamantyl analog **7c** was the most potent inhibitor. Also, compounds containing 3,5-disubstituted phenyl rings (**7j** and **7l**), with the exception of the difluoro compound **7h**, exhibited inhibitory activity, whereas the corresponding 2,4-disubstituted analogs (**7i** and **7k**) did not. These data represent three independent experiments performed in duplicate. The assay is robust and reproducible, and significantly accelerated the testing of this library.

We subsequently took the six lead compounds and determined their IC_{50} values for human Icmt using the methanol vapor diffusion assay[11,20] and the IC_{50} values are shown in Table 1. As in the SPA above, the adamantyl analog **7c** was the most potent inhibitor with the lowest IC_{50}. We are currently synthesizing a more comprehensive library to further explore both the substrate and inhibitory requirements for prenylcysteine binding to Icmt.

In summary, we have synthesized a small library of amide-modified farnesylcysteine analogs and screened them for inhibitory activity. We have identified inhibitors of Icmt that are both easily synthesized and potentially more useful than AFC for further biochemical and cellular studies. These compounds are much more potent inhibitors of human Icmt than our previously reported prenyl-modified analogs such as **2**, although not as potent as inhibitor **3**. We are now in a position to utilize this information to develop more potent inhibitors of recombinant human Icmt.

Acknowledgments

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