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

James L. Donelson^a, Heather B. Hodges^b, Daniel D. MacDougall^c, Brian S. Henriksen^a, Christine A. Hrycyna^{b,*}, and Richard A. Gibbs^{a,*}

^aDepartment of Medicinal Chemistry and Molecular Pharmacology and the Purdue Cancer Center, Purdue University, West Lafayette, IN 47907, USA

^bDepartment of Chemistry and the Purdue Cancer Center, Purdue University, West Lafayette IN 47907, USA

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-member library of compounds, six inhibitors were identified and evaluated further. The adamantyl derivative **7c** was the most potent inhibitor with an IC₅₀ 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 (Figure 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 tumors⁴ due to alternative geranylgeranylation of Ras in FTI treated cells.^{5,6}

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, Figure 2, **1**) in hopes of developing potent anticancer agents as well as molecular tools to study the structure and mechanism of Icmt.

Recently reported work from our laboratories has shown that selective changes in the farnesyl group of AFC can afford effective inhibitors of yeast Icmt (Figure 2, **2**; $K_I = 17 \mu\text{M}$).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** Figure 2; $\text{IC}_{50} = 2.4 \mu\text{M}$ in vitro), resulted in an Icmt-dependant 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 (Figure 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*-farnesyl-thioacetic 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 ($\text{IC}_{50} = 2.8$ and $4.6 \mu\text{M}$, respectively),^{13,14} but these studies were performed with crude membrane preparations from tissues rather than the well-characterized recombinant human Icmt¹¹ 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 pleiotropic 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 iPr_2EtN . In some instances this solution required heating to dissolve the carboxylic acid prior to addition of **5**. After 2–3 hours 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 *S. cerevisiae* cells overexpressing active human Icmt were incubated with $20 \mu\text{M}$ of the methyl donor *S*-adenosyl-[^{14}C -methyl]methionine (SAM) and $25 \mu\text{M}$ 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).

From our initial screen of twenty-three compounds, six agents (**7c**, **7e**, **7j**, **7l**, **7s**, **7w**) led to a 50% or greater decrease in BFC methylation, our criteria for advancing a compound into additional testing (Figure 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 IC₅₀ values for human Icmt using the methanol vapor diffusion assay^{11,20} and the IC₅₀ values are shown in Table 1. As in the SPA above, the adamantyl analog **7c** was the most potent inhibitor with the lowest IC₅₀. 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.

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References and Notes

- (1). Reid TST, KL, Casey PJ, Beese LS. *Journal of Molecular Biology*. 2004; 342:417. [PubMed: 15451670]
- (2). Adjei AA, Erlichman C, Davis JN, Cutler DL, Sloan JA, Marks RS, Hanson LJ, Svingen PA, Atherton P, Bishop WR, Kirschmeier P, Kaufmann SH. *Cancer Res*. 2000; 60:1871. [PubMed: 10766174]
- (3). Zujewski J, Horak ID, Bol CJ, Woestenborghs R, Bowden C, End DW, Piotrovsky VK, Chiao J, Belly RT, Todd A, Kopp WC, Kohler DR, Chow C, Noone M, Hakim FT, Larkin G, Gress RE, Nussenblatt RB, Kremer AB, Cowan KH. *J. Clin. Oncol*. 2000; 18:927. [PubMed: 10673536]
- (4). Johnston SR, Kelland LR. *Endocr Relat Cancer*. 2001; 8:227. [PubMed: 11566614]
- (5). Rowell CA, Kowalczyk JJ, Lewis MD, Garcia AM. *J. Biol. Chem*. 1997; 272:14093. [PubMed: 9162034]
- (6). Whyte DB, Kirschmeier P, Hockenberry TN, Nunez-Oliva I, James L, Catino JJ, Bishop WR, Pai J-K. *J. Biol. Chem*. 1997; 272:1445.
- (7). Winter-Vann AM, Casey PJ. *Nat Rev Cancer*. 2005; 5:405. [PubMed: 15864282]
- (8). Bergo MO, Leung GK, Ambroziak P, Otto JC, Casey PJ, Gomes AQ, Seabra MC, Young SG. *Journal of Biological Chemistry*. 2001; 276:5841. [PubMed: 11121396]
- (9). Michaelson D, Ali W, Chiu VK, Bergo M, Silletti J, Wright L, Young SG, Philips M. *Mol. Biol. Cell*. 2005; 16:1606. [PubMed: 15659645]

- (10). deSolms SJ, Ciccarone TM, MacTough SC, Shaw AW, Buser CA, Ellis-Hutchings M, Fernandes C, Hamilton KA, Huber HE, Kohl NE, Lobell RB, Robinson RG, Tsou NN, Walsh ES, Graham SL, Beese LS, Taylor JS. *J. Med. Chem.* 2003; 46:2973. [PubMed: 12825937]
- (11). Anderson JL, Henriksen BS, Gibbs RA, Hrycyna CA. *J. Biol. Chem.* 2005; 280:29454. [PubMed: 15946942]
- (12). Winter-Vann AM, Baron RA, Wong W, dela Cruz J, York JD, Gooden DM, Bergo MO, Young SG, Toone EJ, Casey PJ. *PNAS.* 2005; 102:4336. [PubMed: 15784746]
- (13). Marciano D, Ben-Baruch G, Marom M, Egozi Y, Haklai R, Kloog Y. *J. Med. Chem.* 1995; 38:1267. [PubMed: 7731012]
- (14). Perez-Sala D, Gilbert BA, Rando RR, Canada FJ. *FEBS Letters.* 1998; 426:319. [PubMed: 9600259]
- (15). Ma, YT.; Gilbert, BA.; Rando, RR. *Methods in Enzymology.* Casey, P.; Buss, J., editors. Vol. Vol. 250. Academic Press; New York: 1995. p. 226
- (16). Ma YT, Shi YQ, Lim YH, McGrail SH, Ware JA, Rando RR. *Biochemistry.* 1994; 33:5414. [PubMed: 8180165]
- (17). Brown MJ, Milano PD, Lever DC, Epstein WW, Poulter CD. *J. Am. Chem. Soc.* 1991; 113:3176.
- (18). Whyte, D.; McGuirk, M.; Nunez-Oliva, I.; Hockenberry, T.; Pai, J. US Patent. Schering-Plough; US: 2001. b) Reactions are carried out in a 96-well microplate. Each well consists of 15 μ g crude membrane protein expressing human Icmt, 11 25 μ M BFC, 20 μ M S-adenosyl-L-[14 C-methyl] methionine (SAM, 50–60 mCi/mmol), and 100 mM Tris-HCl buffer pH 7.5. The appropriate concentration of inhibitor dissolved in DMSO was also added in 2 μ L aliquots to the corresponding well. The plate was then sealed and gently vortexed four times. The plate was incubated at 37 °C for 1 h, and 300 μ g streptavidin SPA beads were added and mixed by pipetting. After 3h at rt the plate was counted in a TopCount scintillation counter..
- (19). A closely related compound, N-biotinyl-S-farnesyl-L-cysteine, was recently utilized by Baron and Casey in a different Icmt assay: Baron RA, Casey PJ. *BMC Biochem.* 2004; 5:19. [PubMed: 15625008]
- (20). Hrycyna CA, Wait SJ, Backlund PS, Michaelis S. *Methods Enzymol.* 1995; 250:251. [PubMed: 7651156]

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

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

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

Scheme 1.

a) Farnesyl-Cl, 7N NH₃/MeOH, 0 °C to rt, 82% (b) R-COOH, HOBt, EDC, DIEA, DMF, rt, 50–95% c) NaOH, 95% EtOH/H₂O, rt, 40–75%

Table 1

Inhibition of Human Icmt by Selected Compounds.

Compds	% of Control ^{a,c}	Enzyme Inhibition IC ₅₀ , μ M ^{a,d}
None	100.0 ($\pm$ 19.0)	NA ^b
7c	12.8 ($\pm$ 7.9)	12.4 ($\pm$ 1.0)
7e	50.1 ($\pm$ 3.5)	16.6 ($\pm$ 1.3)
7j	33.5 ($\pm$ 4.3)	23.0 ($\pm$ 1.9)
7l	37.1 ($\pm$ 4.2)	14.3 ($\pm$ 0.8)
7s	35.1 ($\pm$ 2.2)	17.4 ($\pm$ 1.6)
7w	28.0 ($\pm$ 2.9)	20.3 ($\pm$ 2.6)

^aValues are means of three experiments, SEM, is given in parentheses.^bNA= not applicable.^cDetermined using an SPA procedure.^{18b}^dIC₅₀ values determined using vapor diffusion assay²⁰ and calculated using GraphPad Prism 4.0