

9

Inhibitors of Postprenylation CAAAX Processing Enzymes

JOEL A. BERGMAN^a • JAIMEEN D. MAJMUDAR^a •
RICHARD A. GIBBS^a • CHRISTINE A. HRYCYNA^b

^a*Department of Medicinal Chemistry and Molecular Pharmacology and the Purdue University Center for Cancer Research, Purdue University West Lafayette, Indiana, USA*

^b*Department of Chemistry and the Purdue University Center for Cancer Research, Purdue University West Lafayette, Indiana, USA*

I. Abstract

Eukaryotic signaling proteins, such as the oncogenic Ras proteins, that possess a C-terminal –CaaX sequence undergo a sequential three-step modification pathway consisting of cysteine prenylation, –aaX proteolysis, and prenylcysteine carboxyl methylation. Inhibition of any one of these steps can result in improper protein localization and curtailed protein function, thus it would be desirable to develop inhibitors of these steps as potential therapeutic agents. Potent inhibitors of the prenylation step proved to lack efficacy when evaluated against Ras-driven tumors; therefore, attention was then focused on the two downstream postprenylation events as potential therapeutic targets. Ras-converting enzyme 1 (Rce1) and isoprenylcysteine carboxyl methyltransferase (Icmt) are currently being investigated as targets for such malignancies. In this review, inhibitors of these postprenylation enzymes are described.

II. Introduction

The Ras superfamily comprises a set of G-protein-coupled proteins that undergo three necessary posttranslational modification steps. Oncogenic Ras has been implicated in a number of human malignancies, including many colon and pancreatic cancers that are refractory to chemotherapy [1]. In order for Ras to undergo proper localization to the plasma membrane (PM) for functional activity, it must be posttranslationally modified at its C-terminus by three sequential enzymatic steps. These sequential modifications include protein prenylation, proteolysis, and methyl esterification and are signaled by the presence of a $-CaaX$ motif, where C is cysteine, “a” is generally an aliphatic residue, and X is one of a number of amino acids. Additionally, some Ras proteins are also palmitoylated [2]. The first step is the attachment of an isoprenyl lipid at the cysteine of this $-CaaX$ sequence. Subsequently, the endoprotease Rce1 cleaves the tripeptide $-aaX$ residues, exposing a free carboxylate on the prenylated cysteine residue. The final common step is the methylation of the α -carboxyl of the cysteine residue, catalyzed by Icmt. The summation of these steps results in a gain in hydrophobicity that aids in membrane association of the target protein [3]. Therefore, pharmacologic modulation of these necessary steps may lead to protein mislocalization and ultimately disruption of protein function.

The link between Ras and cancer prompted intensive efforts to block the activity of Ras. Since the initial prenylation is critical to the biological activity of Ras, the prenyltransferases became natural early targets as potential therapeutics. Farnesyltransferase (FTase) quickly became the target of choice, and many small molecule inhibitors of the enzyme were reported in both the pharmaceutical industry and academia. However, clinical trials with potent FTase inhibitors (FTIs) proved to be unsuccessful for patients with pancreatic cancer [4], non-small cell lung cancer [5], small cell lung cancer [6], and urothelial cell carcinoma [7]. Additionally, preclinical trial data for FTIs in N- and K-Ras murine tumor models did not display the desired tumor regression [8,9]. One prime reason for the failure of the FTIs is an alternative prenylation event, where the cysteine that is normally farnesylated is instead geranylgeranylated by geranylgeranyltransferase I. As both Rce1 and Icmt recognize both geranylgeranylated and farnesylated substrates, the downstream processing and subsequent membrane localization of these proteins are not negatively affected, partially accounting for the poor response of tumors to FTIs [10,11]. This lack of efficacy decreased enthusiasm for the FTIs as cancer therapeutics and shifted the focus to the downstream protease and methyltransferase enzymes in the processing pathway.

III. Inhibitors of Rce1

A. DISCOVERY OF RCE1

The first enzymatic modification that occurs after prenylation is the endoproteolysis of the last three amino acids in the $-CaaX$ sequence. Evidence for this proteolysis came from studying modifications to CaaX proteins p21^{ras} [12] and the yeast **a**-mating factor [13]. Nearly a decade later, the genes for Rce1 were identified in multiple organisms and Rce1 was described as a putative membrane-associated metalloproteinase localized to the ER of eukaryotic cells [14,15]. However, conflicting reports suggest that Rce1 may act as a cysteine protease and thus complete elucidation of the exact mechanism of action awaits further study. Rce1 is also thought to be critical for cell viability because Rce1^{-/-} animals display embryonic lethality [16]. Rce1 is capable of processing both farnesylated and geranylgeranylated proteins [17] and as such would avoid the pitfalls encountered with FTIs, as described above. Thus, these data led researchers to investigate Rce1 as a potential therapeutic target to block Ras activation.

B. SUBSTRATE SPECIFICITY OF RCE1

Rando and coworkers pioneered studies to characterize the substrate specificity of the Rce1 endoprotease. Prenylated tetrapeptides were found to be substrates of the enzyme and presence of the lipid conjugation was found to be a requisite functionality [18]. Additionally, a preference for the natural amino acid isomers was also observed as reversal of the chiral center proved detrimental to enzymatic turnover, pointing to a potential role for substrate recognition in the region C-terminal to the scissile bond. These findings were key to not only providing an initial model substrate but also offering a potential starting point for inhibitor design.

Poulter and Rine carried out an extensive investigation into the substrate specificity in the two yeast splice variants of the protease, Rce1 and Afc1 [19]. The authors generated a $-CaaX$ library originating from the **a**-factor oligopeptide, with modifications at each of the a₁, a₂, and X positions. For each of the splice variants, there was a distinct preference for specific residues at each of the positions. Additionally, there were also library members that were capable of only undergoing proteolysis by one of the enzymes. Despite a sequence similarity between the two splice variants, it appeared that they each possessed unique amino acid preferences; however, some overlap was observed. Poulter and coworkers also synthesized CaaX peptides from both yeast and mammalian proteins to further characterize the enzymatic activity of Rce1 [20]. While both farnesylated and

geranylgeranylated oligopeptides could bind to Rce1 and prevent turnover of a radiolabeled substrate, there was a clear preference in terms of catalytic efficiency for farnesylated substrates.

C. RCE1 INHIBITORS

1. Peptide-Based Compounds

Information regarding the substrate activity of Rce1 was central to the development of substrate-based inhibitors. Investigation with farnesylated cysteine and farnesylated tetrapeptides did, however, result in enzymatic inhibition. The carboxylate terminus of a prenylcysteine modified with an aldehyde was found to be a low micromolar inhibitor [21]. Rando and coworkers also investigated modified farnesylated tetrapeptides. By modifying the substrate to remove the scissile bond, the potent statine-tetrapeptide (Figure 9.1) was discovered and has low nanomolar activity against Rce1 in crude membrane fractions.

2. Covalent Modifying Inhibitors

Another class of endoprotease inhibitors was developed that contained α -chloro ketones as the chemical warhead [22]. BFCCMK (Figure 9.1), a simple prenylcysteine analog, presumably operates by an active-site residue displacing the reactive α -chloro ketone. The potency of BFCCMK over nonlipidic small molecules further demonstrates that there are necessary and specific lipophilic motifs for Rce1 binding and inhibition. Pharmacologic

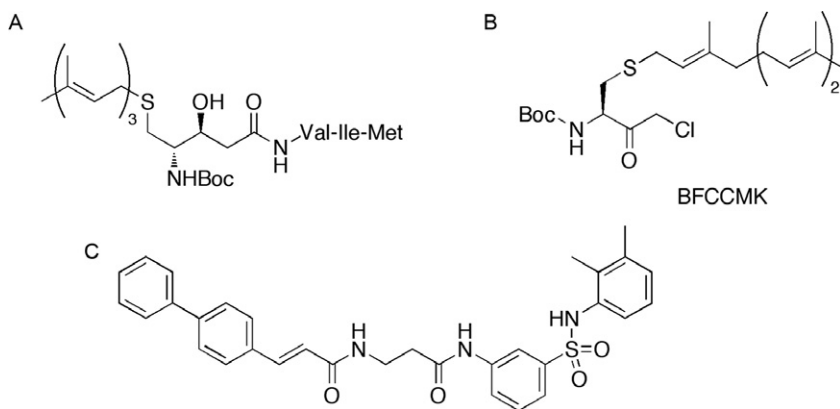


FIG. 9.1. Small molecule inhibitors of Rce1: (A) *N*-Boc-farnesylated tetrapeptide, (B) BFCCMK, (C) a bisubstrate inhibitor.

inhibition with BFCCMK was associated with inhibition of anchorage-independent cell growth in both *K-RAS*-transformed rodent cells and human cancer cells by induction of apoptosis [23].

Recent work from the Schmidt laboratory utilized dipeptide-based acyloxy methyl ketones as the warhead [24,25]. Unlike the aforementioned compounds, these dipeptides utilize a different leaving group; however, presumably enzyme alkylation occurs in a similar fashion. The most potent inhibitors resulted when an Arg residue was positioned proximal to the warhead [25]. However, the authors suggest that inhibition is a result of reversible, noncompetitive binding. This is supported by a “warhead-free” analog maintaining inhibitory activity despite lacking the chemical ability to irreversibly alkylate the active site.

3. *Bisubstrate-Based Approach*

Seeking to overcome the limitations of farnesyl-containing lipopeptides, Casey et al. reported nonprenyl, nonpeptidyl-based analogs with low micromolar potency against Rce1 (C, Figure 9.1) [26]. These analogs were bisubstrate in design as they contained a farnesyl mimetic connected to a peptidomimetic motif, representing a significant advancement toward more drug-like inhibitors of Rce1. It is of particular note that some of the compounds evaluated as Rce1 inhibitors also proved to be weak inhibitors of FTase, most likely attributable to the bisubstrate design. However, they were not capable of inhibiting the final methyl esterification event catalyzed by Icmt. The most potent compounds in these efforts contain a substituted cinnamyl prenyl mimetic affixed to an ablated peptidomimetic of the terminal Met residue of the substrate (farnesyl-CVIM) via a β -Ala linker.

4. *Genetic Models of Rce1*

A murine model with a conditional *Rce1* knockout was found to induce cardiomyopathy and the life span of mice infected with Cre adenovirus to delete *Rce1* was significantly decreased to 10 months [27]. Moreover, it has been shown that Rce1 deficiency actually accelerates the development of myeloproliferative diseases [28]. More recently, a study investigated the postprenylation processing of phosphodiesterase 6 (PDE6) and the impact of ablation of Rce1 activity in neuronal cells [29]. The authors generated mice lacking Rce1 in retinal cells to further elucidate the postprenylation activity for PDE6 and found that Rce1 is necessary for photoreceptor cell viability, and that genetic knockdown reduced the ability for light-evoked responses. Like other proteins requiring postprenylation processing, PDE6 was not properly transported to the correct compartment in *Rce1*^{-/-}

retinas. However, deletion of Rce1 did not detract from proper multimer assembly or enzymatic activity.

D. CONCLUSION FOR RCE1 INHIBITION

The advancement of small molecule inhibitors of Rce1 may be outpaced by the developing story on its functional activity in a variety of cell types, as highlighted briefly above. Inhibition of the postprenylational proteolysis catalyzed by Rce1 as a means to curtail disease states may prove unattainable in light of its unfolding vital functions and thus not efficacious as a therapeutic route. However, the continued evolution of small molecule inhibitors of Rce1 may prove to be useful chemical tools as opposed to therapeutic agents to further elucidate mechanistic and functional activities. A comprehensive review of Rce1 is found in an accompanying chapter in this volume (Chapter 10).

IV. Inhibitors of Icmt

A. DISCOVERY AND CLONING OF YEAST STE14P

The first evidence for the existence of the methylation of a prenylated peptidyl substrate was found in two jelly fungi, *Tremella mesenterica* and *Tremella brasiliensis* [30,31]. These peptide sequences are similar to the **a**-mating factor found in *Saccharomyces cerevisiae*, which is also prenylated at the C-terminus and contains an α -carboxyl methyl ester [32]. The gene product of the *STE14* in *S. cerevisiae* was found to be responsible for the methylation event for the **a**-factor-mating pheromone and the enzymatic activity of this methyltransferase was found only in the cellular membrane fractions [33,34]. Further analyses of Ste14p via epitope tagging determined that the enzyme is localized to the ER membrane and possesses six transmembrane segments, with majority of the enzyme exposed to the cytosol [35,36].

Another milestone development in the story of Ste14p was the report of the purification, liposomal reconstitution, and enzymatic characterization of the methyltransferase from *S. cerevisiae* [37]. Through purification, the authors offered definitive evidence toward Ste14p being the sole component of the enzyme responsible for the methyl esterification of prenylated protein substrates. This report also disclosed that Ste14p was capable of methylating both farnesylated and geranylgeranylated proteins equivalently, further providing evidence for an isoprenyl-recognition motif in the

enzyme. Thus, Ste14p is the founding member of homologous family of methyltransferases known as the Icmts.

B. DISCOVERY AND CLONING OF HUMAN ICMT

The first evidence for this modification in mammalian cells was obtained when human *Ha-Ras* was transfected into rat fibroblasts and was found to be methylated at a C-terminal prenylated cysteine [38]. Based on the previous studies in yeast and jelly fungi, it was suggested this occurred by a third class of methyltransferase. Although Ras proteins have little structural and sequence similarity to the fungi and yeast peptide substrates, they all have a unique characteristic: a prenylated cysteine residue at the carboxyl terminus. The authors thus proposed that this motif is responsible for the recognition of this new class of methyltransferase.

Methyltransferase activity was later identified in both rat liver cells and in bovine retinal rod outer segments [39,40]. Similar to the yeast Icmt, only substrates bearing the prenylation signature were recognized by the enzyme, including both farnesyl and geranylgeranyl moieties [39]. The enzymatic activity was found only in cellular membrane fractions suggesting a similar cellular localization of the Icmt in mammalian cells.

The biochemical reaction performed by the methyltransferase appears to be metal dependent, as determined by chemical inhibition studies, but the exact identity of this metal has yet to be defined [41]. EDTA treatment had no inhibitory effect on Ste14p, but incubation of the protein with 10 – 50 mM of the metal chelating agent 1,10-phenanthroline eliminated catalytic activity [37,41]. Additionally, even more hydrophobic metal sequesterants such as zincon [37], lysine nitriloacetic acid (Lys-NTA) [42], and cholesteryl-Lys-NTA [43] also inhibited the methyltransferase but at much lower concentrations. Together, these data suggest that the metal ion may be buried in a hydrophobic region of the enzyme.

C. PHARMACOLOGIC ACTIVITY OF AFC AND ICMT

Icmt catalyzes the methyl esterification of the prenylated cysteine residue after Rce1 has proteolyzed the –CaaX-containing proteins. The identification of *N*-acetyl-*S*-farnesyl-*L*-cysteine (AFC; Figure 9.2) as a minimal substrate for Icmt was a key event in the development of the Icmt story. The Rando laboratory accomplished much of this pioneering work on Icmt using crude membranes of bovine retinal rod outer segments. In this system, AFC was found to have a K_M of 23 μ M [40]. This finding was instrumental for the pharmacologic modulation of Icmt utilizing small molecules. Treatment with

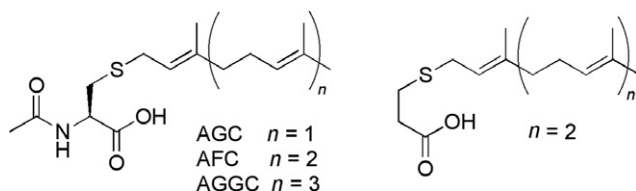


FIG. 9.2. Small molecule farnesylcysteine analogs are substrates of Icmt: Left, *N*-acetyl farnesylcysteine analogs; right, farnesylthiopropionic acid (FTP).

AFC *in vitro* has been shown to prevent the methylation of the prenylpeptide substrate and results in pleiotropic biological consequences.

Several laboratories subsequently studied the effect of AFC treatment on cells. AFC treatment resulted in a decrease of endogenously methylated Ras proteins [44]. The authors also reported that when the isoprenyl-recognition motif was shortened, the binding of the small molecules decreased. Subsequent studies have provided further support for the importance of the isoprenoid motif and hydrophobic bulk. Additional investigation demonstrated that preventing the methylation activity with AFC decreased murine macrophage chemotaxis in a dose-dependent manner, signifying a disruption in a signaling transduction pathway. Further support for disruption of signaling transduction pathways came from Stock and coworkers who demonstrated that AFC prevented aggregation of human platelets upon stimulation with thrombin and collagen, as this process is mediated via G-protein signaling [45]. Additionally, AFC treatment also affected the release of insulin from pancreatic cells, implying that the methylation state of G-proteins is important for their signaling outcomes [46]. AFC was also found to disrupt the Ras/Raf/MEK/ERK pathway at the Ras level in the signaling pathway [47,48]. In this study, the authors used GFP-Ras constructs to determine if Icmt inhibition could prevent translocation to the PM. Again, it was clear that the isoprenyl group in AFC was vital, as decreasing the lipid length diminished the observed cellular activity. Last, AFC was also found to block superoxide release in human neutrophils [49]. Interestingly, modification to the acyl group in AFC could either inhibit or induce superoxide release. This early finding provided an early clue to the structure–activity relationships (SARs) for Icmt substrate binding.

Together, these data suggest that altering the methylation state of protein substrates (or alternatively disrupting methylated-prenylprotein interactions [50]) by Icmt inhibitors may be the cause for these wide-ranging effects. However, it should be noted that AFC is not specific for Icmt and these observations await confirmation with *bona fide* inhibitors that are specific for Icmt.

D. INHIBITORS OF ICMT

1. Prenylcysteine Requirements for Inhibition

In order to develop small molecules capable of eliciting similar effects as AFC in the pharmacologic inhibition of Icmt, it has thus become evident that specific structural requirements must be observed. Specifically, the isoprenyl motif was identified as a necessary recognition group and the presence of a correct prenyl moiety is a key feature for biological activity. Thus, pharmacophore development for Icmt inhibitors became the focus of several research groups in the coming years.

Icmt catalyzes the methyl esterification of the prenylated cysteine residue after Rce1 has proteolyzed the -CaaX-containing proteins. The first step in identification of the minimal substrate for Icmt was through identification of AFC (Figure 9.2) as described above. Interestingly, farnesylcysteine (FC), which is devoid of the acetyl substitution, was not a substrate but did possess some activity as an inhibitor [51], suggesting that the free amine of FC requires modification for catalytic turnover. Alterations in the stereochemistry about the FC backbone also appeared to be detrimental to substrate activity. The stereoisomer, D-AFC, was not a substrate for Icmt but was a modest mixed-type inhibitor of the enzyme. AFC-methyl ester (AFC-Me) was also reported to be a mixed-type inhibitor with respect to both L-AFC and S-adenosylmethionine (SAM), the methyl donor, with K_I values of 41 and 73 μM , respectively [52,53]. The farnesyl homocysteine homolog of AFC is not a substrate for the enzyme; however, the racemic DL-homocysteine farnesyl derivative is in fact a weak inhibitor [40]. Similar to the results with racemic prenylcysteine, these data demonstrate that the linker between the carboxylate and thioether moieties is critical for substrate activity.

Although AFC is a minimal substrate containing some peptide-like character, it is not the minimal substrate for the enzyme. Omission of the acetimido motif results in S-farnesyl thiopropionic acid (FTP; Figure 9.2), which is a substrate for Icmt with a K_M of 14 μM , comparable to that of AFC [40]. These data suggest the acetamido of AFC is not a requisite molecular framework for substrate activity, as the free amino FC is an inhibitor. The efficient turnover of FTP by Icmt has important consequences for substrate-based inhibitor design, as it simplifies analog design by eliminating the stereogenic center, lowers the molecular weight, and increases ligand efficiency [54].

Apart from the cysteine backbone and carboxylate motifs, the prenyl chain also has a major role in enzyme recognition. The C₁₀ geranyl analog of AFC (AGC) is a very poor substrate with approximately 40-fold lower substrate activity [40]. In contrast, replacement with a C₂₀ geranylgeranyl

motif (AGGC) restores substrate activity. In fact, Icmt recognizes and turns over molecules containing both farnesyl and geranylgeranyl moieties equivalently [37]. These data indicate that an isoprene moiety of a certain minimal length is requisite for recognition by the enzyme. Further evidence for this comes from a saturated-isoprene AFC analog (ATC) that is surprisingly devoid of enzymatic activity [40]. Not only are the isoprene units necessary in these substrate-based analogs, but also simple saturated mimetics are apparently not recognized by the enzyme. These results may point to specific interactions within a prenyl-binding pocket between the substrate and the enzyme active site. Taken together, the presence of specific lipid structures is greatly favored for enzyme recognition, and successful inhibitors need to be designed with sufficient isoprene character incorporated.

2. *Heteroatom Requirements of AFC-Based Analogs*

Altering the sulfur heteroatom in AFC and FTP presented an opportunity not only to gain potential mechanistic insights into the methyltransferase but also to develop SAR for inhibitor design. Much of this preliminary work was pioneered in the Rando laboratory and focused on replacing the thioether of AFC and FTP with other heteroatoms. Oxygen- and selenium-substitution resulted in substrates, albeit with greatly reduced turnover [55]. It is interesting to note that the oxy-FTP analog maintained a comparable K_M to FTP; however, the V_{max} was greatly diminished. Similarly, an amino-FTP analog was found to be neither a substrate nor an inhibitor. This result could be attributed to a protonated secondary amine at physiological conditions thus affecting enzyme recognition.

Thioethers possess a high oxidation potential and the oxidized sulfoxide-AFC variant is a modestly potent competitive inhibitor with a K_I of 13 μM in crude membranes [40]. As a natural continuation of these studies, Gibbs and Hrycyna designed a desthio-AFC analog, which was hypothesized to possess increased stability and thereby represent an interesting direction in inhibitor design [56]. Unfortunately, both carbon for sulfur analogs, synthesized as a racemic mixture, were devoid of both enzymatic substrate and inhibitory activity. Therefore, it is evident that the thioether is necessary for optimal activity.

More recently, an approach to replace the thioether in FTP with a triazole was attempted utilizing a dipolar cycloaddition to join the prenyl and carboxylate groups [57]. These analogs are not substrates for Icmt, but all possess some activity as inhibitors. Despite the dramatic shift from the thioether to the triazole, the most potent compound retains significant binding affinity and is able to reduce Icmt activity by approximately 50%

at 10 μM . The SAR of these compounds demonstrates an optimal amount of requisite flexibility for inhibition on both sides of this new thioether replacement. Unfortunately, the most potent analogs required increased isoprene lipid tails and represent less than ideal drug-like character for further development. While most of the heteroatom replacements proved to be unsuccessful inhibitors, these results further established the important role of the thioether for the future design of inhibitors of Icmt. In the design of substrate-based analogs, these data suggest a critical need to preserve this structural motif. Despite potential drawbacks to inclusion of the thioether, the gains from this recognition motif currently appear to outweigh the limitations.

3. *Non-cysteine Backbone Modifications: FTP and FTS*

A more successful modification to FTP that resulted in a low micromolar inhibitor was accomplished by shortening the linker between the carboxylate and thioether motifs. Rando et al. reported the activity of *S*-farnesyl thioacetic acid (FTA; Figure 9.3) [40]. This modification results in the loss of substrate activity; however, the analog retains enzyme affinity and inhibitory potency. Kinetic analysis revealed that FTA is a competitive inhibitor with respect to FTP and has a K_i of 4.6 μM . This same modification proved compatible in the geranylgeranyl homolog (GGTA). Since Icmt turns over both types of prenylated substrates, it comes as no surprise that the enzyme recognizes this analog, and in fact, it is a relatively potent competitive inhibitor with a K_i of 3.9 μM . The significance of these data foreshadowed future work, as minor modifications to the substrate resulted in potent inhibitors of the enzyme, suggesting that substrate-based efforts could lead to the design of Icmt inhibitors.

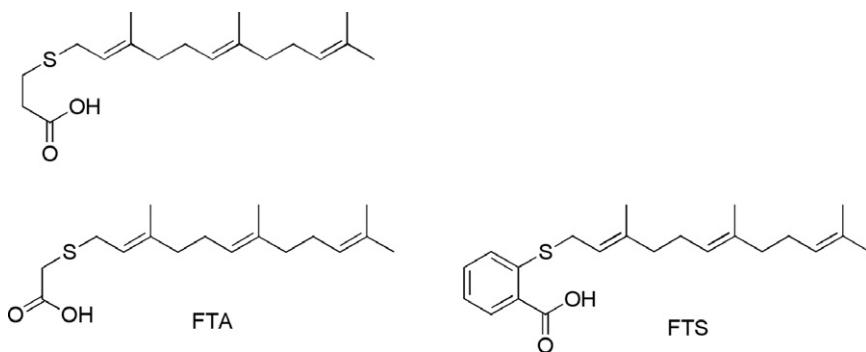


FIG. 9.3. Remodeling of the FTP backbone results in Icmt inhibitors: Left, FTA; right, FTS.

Substitutions extending along the backbone of FTP were also evaluated [51]. Interestingly, substituting an α -methyl adjacent to the carboxylate results in a substrate with a K_M of 20 μM , on par with that of AFC. Intriguingly, substrate activity is maintained when the hybridization is changed from the sp^3 to the sp^2 methylene derivative (FTMA). Conversely, when β -alkyl substitutions to the carboxylate are made, weak inhibitors result. Consequently, these data demonstrate that additions to this region result in modulation of activity depending upon the substitution pattern.

A logical extension of this approach was continued in the Kloog laboratory with the discovery of the small molecule Icmt inhibitor, farnesyl thiosalicylic acid (FTS; Figure 9.3) [58]. FTS maintains the same number of linking atoms as FTP yet has a similar lack of rotational freedom as FTA. This rotational restriction renders FTS a low micromolar inhibitor that has an IC_{50} of 3 μM in a cell-free system.

The discovery of FTS led to an investigation into the SAR of the aromatic backbone in this scaffold. A switch to an amino linkage and altered benzene substitution resulted in a decrease in biochemical activity. Likewise, a 5-amino FTS analog also lost biochemical activity against the methyltransferase [58]. Interestingly, a series of halo-FTS analogs maintained inhibitory activity against Icmt [59]. 5-Chloro and fluoro analogs of FTS have K_I values most closely resembling that of parent FTS. However, moving the halogen to the 3- and 4-positions results in a dramatic loss in biochemical potency. As expected, the prenyl chain continues to play an important role in the activity of FTS and its derivatives. Shortening to the C_{10} geranyl analog diminishes inhibition. Increasing the length to the C_{20} geranylgeranyl analog also decreases activity [59]. The latter finding is unexpected given the fact that GGTA maintains inhibitory activity equivalent with FTA, albeit with increased lipid character.

Although FTS was a potent inhibitor of Icmt in enzymatic assays *in vitro*, it was a weak methyltransferase inhibitor in intact cells [60]. A variety of cells treated with high doses of FTS maintained high levels of methylated proteins, thus indicating proper processing despite inhibitor treatment. Despite this fact, FTS was capable of inhibiting the growth and changing the morphology of Ha-Ras transformed Rat1 cells. The authors concluded that this cellular activity is not likely due to inhibition of postprenylation processing by Icmt, but rather by indirectly affecting the localization of the prenylated Ras protein. These experiments imply that FTS treatment is not specific for Icmt inhibition and that its biological effects are more likely due to inhibition of other protein-protein interactions.

4. *Cysmethynil: A Small Molecule from Screening Efforts*

High-throughput screening has been a valuable route to the discovery of inhibitors, including FTase (Bishop et al., see Chapter 15, Volume 29). Similar to many other therapeutic targets, small molecule inhibitors of Icmt have been discovered by library screening efforts [61]. Cysmethynil was discovered by the Casey group and was the first reported synthetic compound to show inhibitory activity against Icmt from a high-throughput effort (A; Figure 9.8). As such, cysmethynil is one of the Icmt inhibitors not derived from a substrate-based framework. However, inspection of its structural features reveals that the octyl tail and terminal amide could potentiate interactions with the active site akin to substrate-based inhibitors. Kinetic analysis of cysmethynil reveals that it is a time-dependent low micromolar inhibitor of Icmt [62]. In addition, cysmethynil treatment in a MEF model system induces mislocalization of GFP-KRas constructs and blocked anchorage-independent growth triggered by activated Ras in DKOB8 cells. Treatment in PC3 prostate cancer cells with cysmethynil has also been shown to induce autophagic cell death [63]. Recently, Wang et al. have aimed to improve the pharmacokinetic parameters of cysmethynil [64]. SAR studies have developed primary and secondary amino derivatives of the parent indole core that are inhibitors of Icmt. Introduction of these groups into cysmethynil results in analogs that are reported to retain antiproliferative properties. A comprehensive review of cysmethynil and its biological activity is included in this volume (Chapter 11).

5. *Prenyl-Substituted Inhibitors of Icmt*

With little known about the active site of Icmt, a series of farnesyl-modified AFC analogs were designed to investigate a prenyl-binding site within the enzyme. These compounds were evaluated against both the yeast and human variants of Icmt to aid in the characterization of both active sites [65]. This work relied heavily on the synthetic methodology developed in the Gibbs laboratory in their work designing farnesyl pyrophosphate analogs as modulators of FTase activity (Chapter 5). Despite the similarity between the yeast and human variants, substitutions at the 3-position in the farnesyl group interacted with both enzymes differently, suggesting that these homologs have overlapping but distinct substrate specificities. This 3-position is believed to be important for enzyme recognition and may play a major role in active-site binding. Interestingly, one analog with a 3-position modification proved to be an inhibitor of Ste14p but a substrate for human Icmt [65]. In further analog design, an aryl-containing analog replaced the terminal prenyl domain with a biphenyl motif (Figure 9.4). This change resulted in a poor human Icmt inhibitor with an IC_{50} of 259 μ M in a mixed-competitive inhibition mode. This biphenyl analog was the only prenyl-modified AFC analog

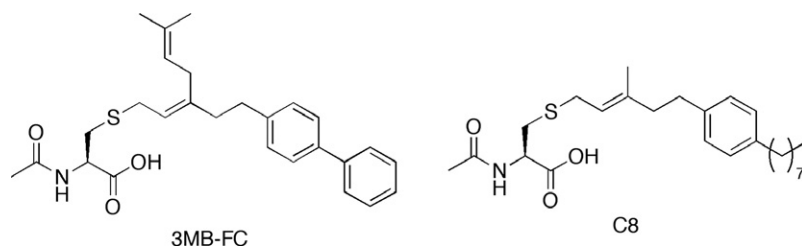


FIG. 9.4. Prenyl-modified AFC analogs are modest inhibitors of Icmt: Left, 3MB-FC; right, alkyl tail C8.

capable of inhibiting variants from both species. A conclusion of that work is that the prenyl recognition motif of human Icmt appears to be more tolerant to substitutions than that of Ste14p. This finding was the impetus for further development of Icmt inhibitors. Since human Icmt is more tolerant to farnesyl substitutions, the development of nonprenyl AFC analogs seemed a possibility in designing more drug-like inhibitors.

As such, a series of compounds were synthesized to find prenyl mimetics with a variety of alkyl and ether replacements for the second and third isoprene groups while maintaining the peptidyl-AFC scaffold [57]. A series of ethers proved to be poor Icmt inhibitors, but an increase in lipidic flexibility led to modest gains in inhibition. More efficient inhibitors were found in a series of alkyl compounds. In these compounds, a trend was observed where increasing the length of the alkyl tail results in improved Icmt inhibition [57]. The most potent analog has a C₈ alkyl tail and an IC₅₀ of 35 μ M (Figure 9.4). Although modest inhibitors, these compounds represent an improvement on two fronts over the previous isoprenoid-based AFC compounds. These compounds reduced the nondrug-like prenyl character of the AFC scaffold and significantly improved inhibitory potency, by nearly 10-fold.

By removing the prenyl character in order to improve drug-likeness, it became evident that certain requirements are critical to maintain in order to achieve Icmt inhibition. Overall length of the prenyl mimetic follows the same requirements of the native lipid, as it appears an increased length is preferred. However, these limited findings do reveal that nonprenyl groups, bearing sufficient hydrophobic bulk, can suffice as farnesyl proxies and occupy the prenylcysteine-binding site as AFC-based inhibitors of Icmt.

6. Amide-Modified FC Analogs

Early work in the Rando laboratory sought to better understand the pleiotropic role of AFC on a variety of cell types by investigating FC derivatives bearing acyl substitutions at the amino motif [66,67].

Amide-modified farnesylcysteine (AMFC) analogs were synthesized and evaluated as substrates or inhibitors of the methyltransferase. Acyl substitutions bearing small alkyl groups are substrates; however, a pivaloyl-FC derivative has neither substrate nor inhibitor activity. A benzoyl-FC series was also investigated and similarly lacked activity; however, introducing a flexible linker between the benzoyl and FC groups restored substrate activity. These data indicate that bulky substitutions at the amine group of the FC are not well tolerated by the enzyme.

An effort was undertaken by the Gibbs and Hrycyna laboratories to use this FC pharmacophore information and expand on it to develop SAR with a wider variety of acyl substitutions. An initial library of 23 AMFCs was synthesized and evaluated for Icmt activity [68]. Three analogs possessed substrate activity and confirmed the previous SAR, as small alkyl moieties and flexible linkers in the amide region led to Icmt substrates [66]. This library resulted in several modestly potent inhibitors of Icmt that possessed bulky substitutions adjacent to the FC backbone. Steric bulk alpha to the amide junction results in more potent inhibitors, with an adamantyl-FC (Figure 9.5) analog having an IC_{50} of 12.4 μ M.

The Gibbs and Hrycyna laboratories then focused on synthesizing analogs of the lead AMFC compound and refining its activity by developing and implementing a solid-phase approach to prenylcysteine analogs [69]. Through this effort, a new *o*-phenoxyphenyl-modified lead was discovered, coined POP-FC (Figure 9.5), that inhibited human Icmt with an IC_{50} of 4.3 μ M. This scaffold is not only more potent than the previous adamantyl-FC lead

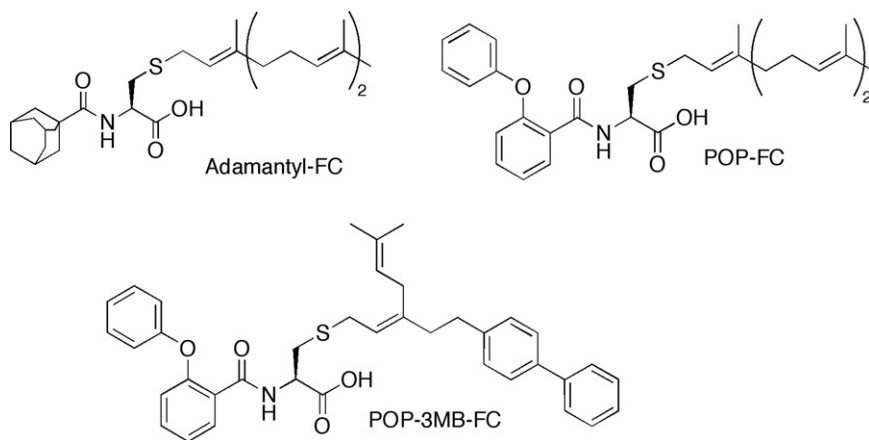


FIG. 9.5. AMFC-based Icmt inhibitors utilize the prenylcysteine recognition motif for potency: Top left, adamantyl-FC; top right, POP-FC; bottom, POP-3MB-FC hybrid.

but also demonstrates an improved starting point for further refinement from a synthetic vantage. These data demonstrate that bulkiness, either in the *o*-phenoxyphenyl or in a quaternary acyl substitution, is key to Icmt inhibition and improves the inhibition profile of AMFC analogs.

Extending this AMFC work, molecules were synthesized that coupled the amide-modified and prenyl-modified halves affording a prenylcysteine hybrid with an IC_{50} of 2.5 μM , termed POP-3MB-FC (Figure 9.5) [69]. This molecule afforded significant increase in potency over the parent AMFC by incorporating the prenyl group of a known Icmt inhibitor [65]. Due to the potency of POP-FC, the SAR of the phenoxyphenyl motif was further refined. This study demonstrated that the *o*-phenoxyphenyl motif is ideal for enzyme inhibition and any deviation results in a loss of potency. This work also clearly demonstrated that the prenyl region of this AMFC pharmacophore is critical for Icmt inhibition [76].

The correct acyl modification results in potent Icmt inhibition, but maintaining the cysteine backbone is not desirable as it results in an unstable allylic thioether. However, simple omission would not suffice for enzymatic recognition. One approach explored was a triazole for sulfur substitution [57]. This approach coupled amide substituents that led to AMFC substrates with a prenylcysteine core via copper-mediated dipolar cycloaddition [70]. Interestingly, this switch resulted in the transformation of Icmt substrates into weak inhibitors. This construction also resulted in reducing the potency of a triazole analog of POP-FC by nearly five times (IC_{50} of 19 μM) versus POP-FC itself. However, these results demonstrated that drastic modifications in the amide linker moiety can be accommodated within the Icmt active site and that this binding site might be more plastic than previously believed [66].

7. Isosteric Replacements in FC Analogs

The pharmacophoric attributes of AMFCs principally contain four molecular scaffolds: (i) prenyl chain, (ii) cysteine backbone, (iii) acetamido, and (iv) carboxylate. Realizing the potential instability of the amide *in vivo*, Gibbs and Hrycyna first attempted the isosteric replacement of the amide by a sulfonamide. This approach was successful in terms of generating a library of analogs that displayed low micromolar inhibition of human Icmt without any examples of substrate behavior, unlike the amide libraries [71]. The most potent sulfonamide-modified farnesylcysteine (SMFC) bore a 2-thienyl modification and has an IC_{50} of 9 μM against human Icmt in crude membrane preparations (Figure 9.6). The findings from this study are notable because it demonstrated that the amide linkage could be altered to a metabolically stable motif while maintaining inhibitory potency. Also

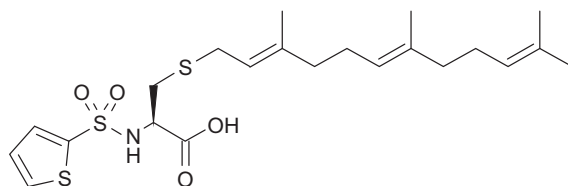


FIG. 9.6. Amide isostere lead thienyl-SMFC is a low micromolar inhibitor of Icmt.

demonstrated was that the stereochemistry of C_{α} is not important for inhibition. Perhaps, the prenyl chain of the inhibitor is able to orient itself favorably within the binding pocket, allowing the analog to bind, despite the varied acyl modifications. This study also strongly suggested that the prenyl motif is the “business end” of the analog and that this motif is what contributes most to enzymatic binding. This hypothesis seems plausible as Icmt is responsible for the methylation of both farnesylated and geranylgeranylated proteins and proteins with varied sequences upstream of the FC motif. Not surprisingly, the sulfonamide bond is a capable surrogate for the amide linkage precisely because the binding pocket is more tolerable to that substitution.

Other isosteric replacements have also been attempted and have resulted in modest Icmt inhibitors, including secondary amines, phosphonamides, carbamates, and triazoles (Jaimeen D. Majmudar, unpublished data). Secondary amines are the weakest inhibitors in this survey. The phosphonamides and triazoles are in fact modest inhibitors, although not as potent as the SMFCs. In this library, the farnesyl lipid chain was held constant further fortifying the hypothesis that the enzyme-binding pocket is more tolerant to substitution when the key lipid recognition motif is present.

It has become increasingly clear that the farnesyl chain and the carboxylate portions of the AFC molecule are the most important for molecular recognition by Icmt. Although much effort focused on identifying a suitable amide isostere and also a possible prenyl mimetic, work at the carboxylate end appears most challenging. Rando et al. synthesized several FTP-modified analogs at the carboxylate terminus [51]. They replaced the carboxylate with a sulfonate and phosphonate as isosteres. Additionally, nitro, amide, and hydroxy-ethanimidothioate were all used to replace the carboxylate moiety. All these efforts resulted in very weak inhibitors suggesting that the terminal carboxylate specifically is critical for analog binding.

Gibbs and Hrycyna have recently shown that replacing the carboxylate of FTA with a primary alcohol results in a submicromolar Icmt inhibitor. This compound has an IC_{50} of 600 nM (Majmudar unpublished data).

However, a majority of other carboxylate replacements resulted in modest inhibitors. These studies demonstrated that while the presence of the carboxylate is important, it may be dispensable and suggest that rational molecular mutagenesis may help achieve an *Icmt* inhibitor that contains a modified carboxylate end.

8. Prenyl Mimetics of FTP

Early efforts clearly demonstrated the lipid requirements for FC analogs modified at both the amide and prenyl regions. However, there was much room for improvement in the development of prenyl mimetics for more drug-like inhibitors of *Icmt*. Gibbs et al. devised an approach to synthesize a library utilizing a dipolar cycloaddition strategy to generate a triazole within the prenyl chain [77]. As such, these compounds were based on the FTP framework by omitting the amide motif and maintaining the identical structure from the carboxylate through the first isoprene, a known key recognition motif. The triazole was positioned in place of the second isoprene, and a variety of terminal alkynes were used to incorporate prenyl tail mimetics.

Rigid aryl groups directly conjugated to the triazole are not efficient inhibitors; however, increasing the methylene linker to give flexibility between the triazole and aromatic moiety resulted in an increase in *Icmt* inhibition. A phenethyl analog has an IC_{50} of 49 μM (Figure 9.7). While this analog is significantly less potent than that of AFMC and SMFC analogs, it has the superior advantage of possessing a higher degree of ligand efficiency [54] and a greater range of medicinal chemistry efforts can be initiated from this early lead.

Because *Icmt* recognizes both farnesylated and geranylgeranylated substrates, it was hypothesized that increasing the overall length by larger aryl groups would be well tolerated by *Icmt* and could lead to increased potency. A biphenyl substitution for the phenyl moiety resulted in a dramatic increase in potency. Moreover, it was found that the methyl ester analog possessed further methyltransferase inhibition. This analog, termed TAB (Figure 9.7), has an IC_{50} of 800 nM and is approximately 30% more potent than the carboxylate analog when evaluated at 10 μM . TAB was evaluated in a MEF cell-culture model system and is cytotoxic only to *Icmt*^{+/+} cells and an in-cell IC_{50} of 33 μM was determined. TAB was also able to induce GFP-KRas mislocalization, further demonstrating *Icmt* inhibition results in reduction of proper KRas functioning. Moreover, TAB was efficacious when evaluated in PaTu cells, a K-Ras-driven pancreatic cancer cell line, with an IC_{50} of 8 μM . This development represents a dramatic

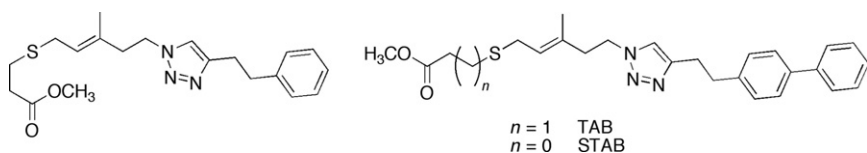


FIG. 9.7. Structures of triazole-based FTP compounds result in potent and selective inhibitors of Icmt: Left, early phenethyl TAB lead; right, biphenyl TAB and STAB.

improvement in substrate-based Icmt inhibitors with improved selectivity to accompany increased potency (Bergman et al., submitted for publication).

Initial medicinal chemistry efforts highlighted an importance of the biphenyl orientation. Further SAR development demonstrated a dramatic increase in potency when the linker between the thioether and carboxylate was shortened by one methylene unit, akin to the FTP–FTA modification. This modification proved more effective in producing potent Icmt inhibition than other modifications occurring within the prenyl motif (Bergman et al., unpublished data). This shortened analog, STAB, is a mixed-competitive Icmt inhibitor with an IC_{50} of 200 nM (Figure 9.7). The free carboxylate analog of STAB again is nearly four times less potent for Icmt inhibition. It is clear in these compounds that the methyl ester is playing a significant role in enzyme inhibition and efforts are currently ongoing to replace the labile methyl ester with a more biocompatible moiety. Further, preliminary *in vitro* evaluation of STAB with the MEFs derived from both wild-type and *Icmt*^{-/-} knockout mice showed an increase in potency as well as selectivity for wild-type cells.

9. Inhibitors of Icmt from Natural Products

Compounds obtained from natural sources have always contributed to the development of small molecules for various targets in the history of medicinal chemistry. Recently, several small molecules derived from natural products have been shown to possess Icmt inhibitory activity [72,73]. This effort to discover other natural product inhibitors of Icmt utilized a HTS approach. Of the approximately 6×10^5 extracts screened, the extract from the order Verongida displayed Icmt inhibition. A bromotyrosine-derived chemotaxonomic marker characterizes marine sponges in this order. The extract from *Pseudoceratina* sp. contained a compound with a bromotyrosyl-spermine-bromotyrosyl sequence, named spermatinamine

(B, Figure 9.8), which displayed Icmt inhibition [72]. It is interesting to note that this chemical sequence is rare in nature and the closest related compounds are philanthoxin and its analogs. These compounds are nonselective antagonists at the ionotropic glutamate receptor and the acetylcholine receptors. Spermatinamine, a symmetrical compound, has an IC_{50} of $1.9 \mu\text{M}$, although the authors did not determine its inhibition kinetics as it is unknown if it is competitive with respect to AFC or SAM or an allosteric modulator. To aid medicinal chemists, a total synthesis of spermatinamine has been reported, thus making the development of analogs to determine its inhibition mechanism easier [74].

Buchanan et al. have also identified another bromotyrosine scaffold containing natural product that possesses Icmt inhibition, named aplysamine 6 (C, Figure 9.8) [73]. Aplysamine 6 is also an alkaloid derived from *Pseudoceratina* sp.; however, it does not have an oxime and is not symmetrical as opposed to spermatinamine. An IC_{50} of $14 \mu\text{M}$ is reported for aplysamine 6, significantly higher than the other extract.

Another set of weak Icmt inhibitors from natural sources were also discovered by Buchanan and coworkers [75]. Prenylated β -hydroxy chalcone derivatives along with the known flavone, (*S*)-glabrol, were discovered from the extract *Hovea parvicalyx* and found to possess Icmt inhibitory activity with an IC_{50} of $17 \mu\text{M}$. Interesting to note that the β -hydroxychalcones have a structural resemblance to some metal chelators. Continued studies on these natural products will further give the medicinal chemist insight into the continued refinement of Icmt inhibitors.

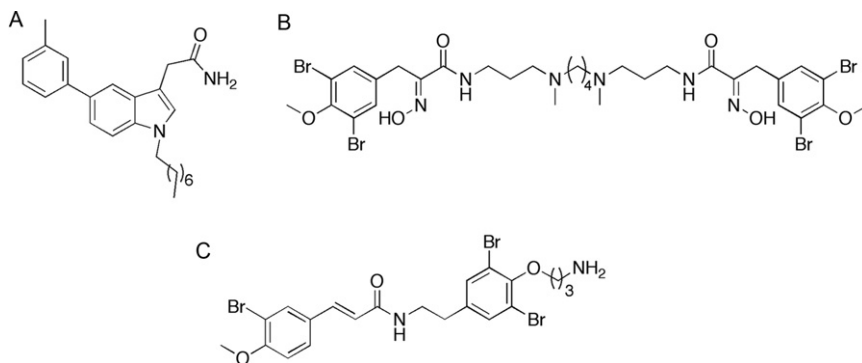


FIG. 9.8. Structures of nonsubstrate-based Icmt inhibitors: (A) cysmethynil, (B) spermatinamine, (C) aplysamine 6.

V. Conclusion

Focusing on the postprenylation enzymes Rce1 and Icmt is an attractive approach for the development of potential therapeutic agents, especially in refractory solid tumors because their enzymatic functions are unique. Inhibitors for the endoprotease have arisen mainly from the design of substrate-based compounds; however, their therapeutic use may be in question because of the recent data indicating the importance of Rce1 function. On the other hand, inhibitors of Icmt demonstrate promise as therapeutic agents.

Progress in the development and evaluation of Icmt inhibitors was painfully slow, particularly in comparison to the rapid development of potent FTIs of diverse classes. There were numerous roadblocks to the development of Icmt inhibitors, including a complete lack of structural knowledge regarding the enzyme, difficulty in purifying and characterizing Icmt, the inherent challenges in working with a membrane-bound enzyme, and the pleiotropic biological effects exhibited by one of the early lead compounds, AFC. However, many of these barriers have been overcome during the past decade. This chapter describes how these advances enabled the development of potent substrate-based Icmt inhibitors with cellular efficacy. We are now at an exciting time in the Icmt field; the availability of nanomolar inhibitors of diverse structural classes (Chapter 11) should allow for several key biological questions to be answered, including those pertaining to the anticancer therapeutic potential of Icmt inhibition.

REFERENCES

1. Downward, J. (2003). Targeting ras signalling pathways in cancer therapy. *Nat Rev Cancer* 3:11–22.
2. Hancock, J.F., Magee, A.I., Childs, J.E., and Marshall, C.J. (1989). All Ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* 57:1167–1177.
3. Michaelson, D., *et al.* (2005). Postprenylation CAAX processing is required for proper localization of ras but not rho GTPases. *Mol Biol Cell* 16:1606–1616.
4. Macdonald, J.S., *et al.* (2005). A phase II study of farnesyl transferase inhibitor R115777 in pancreatic cancer: a Southwest oncology group (SWOG 9924) study. *Invest New Drugs* 23:485–487.
5. Adjei, A.A., *et al.* (2003). Phase II study of the farnesyl transferase inhibitor r115777 in patients with advanced non-small-cell lung cancer. *J Clin Oncol* 21:1760–1766.
6. Heymach, J.V., *et al.* (2004). Phase II study of the farnesyl transferase inhibitor R115777 in patients with sensitive relapse small-cell lung cancer. *Ann Oncol* 15:1187–1193.
7. Rosenberg, J.E., *et al.* (2005). A phase II trial of R115777, an oral farnesyl transferase inhibitor, in patients with advanced urothelial tract transitional cell carcinoma. *Cancer* 103:2035–2041.

8. Mangués, R., *et al.* (1998). Antitumor effect of a farnesyl protein transferase inhibitor in mammary and lymphoid tumors overexpressing N-ras in transgenic mice. *Cancer Res* 58:1253–1259.
9. Omer, C.A., *et al.* (2000). Mouse mammary tumor virus-Ki-rasB transgenic mice develop mammary carcinomas that can be growth-inhibited by a farnesyl : protein transferase inhibitor. *Cancer Res* 60:2680–2688.
10. Whyte, D.B., *et al.* (1997). K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. *J Biol Chem* 272:14459–14464.
11. Zhang, F.L., *et al.* (1997). Characterization of Ha-Ras, N-Ras, Ki-Ras4A, and Ki-Ras4B as in vitro substrates for farnesyl protein transferase and geranylgeranyl protein transferase type I. *J Biol Chem* 272:10232–10239.
12. Gutierrez, L., Magee, A.I., Marshall, C.J., and Hancock, J.F. (1989). Post-translational processing of P21ras is 2-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *EMBO J* 8:1093–1098.
13. Ashby, M.N., King, D.S., and Rine, J. (1992). Endoproteolytic processing of a farnesylated peptide in vitro. *Proc Natl Acad Sci USA* 89:4613–4617.
14. Schmidt, W.K., Tam, A., Fujimura-Kamada, K., and Michaelis, S. (1998). Endoplasmic reticulum membrane localization of Rcep1 and Ste24p, yeast proteases involved in carboxyl-terminal CAAX protein processing and amino-terminal a-factor cleavage. *Proc Natl Acad Sci USA* 95:11175–11180.
15. Otto, J.C., Kim, E., Young, S.G., and Casey, P.J. (1999). Cloning and characterization of a mammalian prenyl protein-specific protease. *J Biol Chem* 274:8379–8382.
16. Kim, E., *et al.* (1999). Disruption of the mouse Rce1 gene results in defective Ras processing and mislocalization of Ras within cells. *J Biol Chem* 274:8383–8390.
17. Jang, G.F., and Gelb, M.H. (1998). Substrate specificity of mammalian prenyl protein-specific endoprotease activity. *Biochemistry* 37:4473–4481.
18. Ma, Y.T., Chaudhuri, A., and Rando, R.R. (1992). Substrate specificity of the isoprenylated protein endoprotease. *Biochemistry* 31:11772–11777.
19. Trueblood, C.E., Boyartchuk, V.L., Picologlou, E.A., Rozema, D., Poulter, C.D., and Rine, J. (2000). The CaaX proteases, Afc1p and Rce1p, have overlapping but distinct substrate specificities. *Mol Cell Biol* 20:4381–4392.
20. Dolence, J.M., Steward, L.E., Dolence, E.K., Wong, D.H., and Poulter, C.D. (2000). Studies with recombinant *Saccharomyces cerevisiae* CaaX prenyl protease Rcep1. *Biochemistry* 39:4096–4104.
21. Ma, Y.T., Gilbert, B.A., and Rando, R.R. (1993). Inhibitors of the isoprenylated protein endoprotease. *Biochemistry* 32:2386–2393.
22. Chen, Y.L., Ma, Y.T., and Rando, R.R. (1996). Solubilization, partial purification, and affinity labeling of the membrane-bound isoprenylated protein endoprotease. *Biochemistry* 35:3227–3237.
23. Chen, Y. (1998). Inhibition of K-ras-transformed rodent and human cancer cell growth via induction of apoptosis by irreversible inhibitors of Ras endoprotease. *Cancer Lett* 131:200.
24. Porter, S.B., Hildebrandt, E.R., Breevoort, S.R., Mokry, D.Z., Dore, T.M., and Schmidt, W.K. (2007). Inhibition of the CaaX proteases Rce1p and Ste24p by peptidyl (acyloxy)methyl ketones. *Biochim Biophys Acta Mol Cell Res* 1773:853–862.
25. Dechert, A.-M.R., *et al.* (2010). Modulation of the inhibitor properties of dipeptidyl (acyloxy)methyl ketones toward the CaaX proteases. *Bioorg Med Chem* 18:6230–6237.
26. Schlitzer, M., Winter-Vann, A., and Casey, P.J. (2001). Non-peptidic, non-prenylic inhibitors of the prenyl protein-specific protease Rce1. *Bioorg Med Chem Lett* 11:425–427.
27. Bergo, M.O., *et al.* (2004). On the physiological importance of endoproteolysis of CAAX proteins—heart-specific RCE1 knockout mice develop a lethal cardiomyopathy. *J Biol Chem* 279:4729–4736.

28. Wahlstrom, A.M., *et al.* (2007). Rce1 deficiency accelerates the development of K-RAS-induced myeloproliferative disease. *Blood* 109:8.
29. Christiansen, J.R., Kolandaivelu, S., Bergo, M.O., and Ramamurthy, V. (2011). RAS-converting enzyme 1-mediated endoproteolysis is required for trafficking of rod phosphodiesterase 6 to photoreceptor outer segments. *Proc Natl Acad Sci USA* 108:8862–8866.
30. Sakagami, Y., Yoshida, M., Isogai, A., and Suzuki, A. (1981). Peptidal sex hormones inducing conjugation tube formation in compatible mating-type cells of *Tremella mesenterica*. *Science* 212:1525–1527.
31. Ishibashi, Y., Sakagami, Y., Isogai, A., and Suzuki, A. (1984). Structures of tremogens A-9291-I and A-9291-VIII: peptidal sex hormones of *Tremella brasiliensis*. *Biochemistry* 23:1399–1404.
32. Anderegg, R.J., Betz, R., Carr, S.A., Crabb, J.W., and Duntze, W. (1988). Structure of *Saccharomyces cerevisiae* mating hormone a-factor. Identification of S-farnesyl cysteine as a structural component. *J Biol Chem* 263:18236–18240.
33. Hrycyna, C.A., and Clarke, S. (1990). Farnesyl cysteine C-terminal methyltransferase activity is dependent upon the STE14 gene product in *Saccharomyces cerevisiae*. *Mol Cell Biol* 10:6.
34. Hrycyna, C.A., Sapperstein, S.K., Clarke, S., and Michaelis, S. (1991). The *Saccharomyces cerevisiae* STE14 gene encodes a methyltransferase that mediates C-terminal methylation of a-factor and Ras proteins. *EMBO J* 10:1699–1709.
35. Romano, J.D., Schmidt, W.K., and Michaelis, S. (1998). The *Saccharomyces cerevisiae* prenylcysteine carboxyl methyltransferase Ste14p is in the endoplasmic reticulum membrane. *Mol Biol Cell* 9:2231–2247.
36. Romano, J.D., and Michaelis, S. (2001). Topological and mutational analysis of *Saccharomyces cerevisiae* Ste14p, founding member of the isoprenylcysteine carboxyl methyltransferase family. *Mol Biol Cell* 12:1957–1971.
37. Anderson, J.L., Frase, H., Michaelis, S., and Hrycyna, C.A. (2005). Purification, functional reconstitution, and characterization of the *Saccharomyces cerevisiae* isoprenylcysteine carboxylmethyltransferase Ste14p. *J Biol Chem* 280:7336–7345.
38. Clarke, S., Vogel, J.P., Deschenes, R.J., and Stock, J. (1988). Posttranslational modification of the Ha-Ras oncogene protein: evidence for a 3rd class of protein carboxyl methyltransferases. *Proc Natl Acad Sci USA* 85:4643–4647.
39. Stephenson, R.C., and Clarke, S. (1990). Identification of a C-terminal protein carboxyl methyltransferase in rat liver membranes utilizing a synthetic farnesyl cysteine-containing peptide substrate. *J Biol Chem* 265:16248–16254.
40. Tan, E.W., Perezsala, D., Canada, F.J., and Rando, R.R. (1991). Identifying the recognition unit for G-protein methylation. *J Biol Chem* 266:10719–10722.
41. Desrosiers, R.R., Nguyen, Q.T., and Béliveau, R. (1999). The carboxyl methyltransferase modifying G proteins is a metalloenzyme. *Biochem Biophys Res Commun* 261:7.
42. Schmitt, L., Dietrich, C., and Tampe, R. (1994). Synthesis and characterization of chelator-lipids for reversible immobilization of engineered proteins at self-assembled lipid interfaces. *J Am Chem Soc* 116:8485–8491.
43. Hodges, H.B., Zhou, M.K., Haldar, S., Anderson, J.L., Thompson, D.H., and Hrycyna, C.A. (2005). Inhibition of membrane-associated methyltransferases by a cholesterol-based metal chelator. *Bioconjug Chem* 16:490–493.
44. Volker, C., *et al.* (1991). Effects of farnesylcysteine analogs on protein carboxyl methylation and signal transduction. *J Biol Chem* 266:21515–21522.
45. Akbar, Huzoor, Wang, W.J., Kornhauser, R., Volker, C., and Stock, J.B. (1993). Protein prenylcysteine analog inhibits agonist receptor-mediated signal transduction in human platelets. *Proc Natl Acad Sci USA* 90:868–872.

46. Kowluru, A., *et al.* (1996). Glucose- and GTP-dependent stimulation of the carboxyl methylation of CDC42 in rodent and human pancreatic islets and pure beta cells. Evidence for an essential role of GTP-binding proteins in nutrient-induced insulin secretion. *J Clin Invest* 98:540–555.
47. Choy, E., *et al.* (1999). Endomembrane trafficking of Ras: the CAAX motif targets proteins to the ER and Golgi. *Cell* 98:69–80.
48. Chiu, V.K., *et al.* (2004). Carboxyl methylation of Ras regulates membrane targeting and effector engagement. *J Biol Chem* 279:7346–7352.
49. Ding, J.B., *et al.* (1994). Farnesyl-L-cysteine analogs can inhibit or initiate superoxide release by human neutrophils. *J Biol Chem* 269:16837–16844.
50. Kloog, Y., and Cox, A.D. (2004). Prenyl-binding domains: potential targets for Ras inhibitors and anti-cancer drugs. *Semin Cancer Biol* 14:61.
51. Gilbert, B.A., Tan, E.W., Perez-Sala, D., and Rando, R.R. (1992). Structure activity studies on the retinal rod outer segment isoprenylated protein methyltransferase. *J Am Chem Soc* 114:3966–3973.
52. Baron, R., and Casey, P. (2004). Analysis of the kinetic mechanism of recombinant human isoprenylcysteine carboxylmethyltransferase (Icmt). *BMC Biochem* 5:19.
53. Shi, Y.Q., and Rando, R.R. (1992). Kinetic mechanism of isoprenylated protein methyltransferase. *J Biol Chem* 267:9547–9551.
54. Abad-Zapatero, C., and Metz, J.T. (2005). Ligand efficiency indices as guideposts for drug discovery. *Drug Discov Today* 10:9.
55. Tan, E.W., Perezsala, D., and Rando, R.R. (1991). Heteroatom requirements for substrate recognition by GTP-binding protein methyltransferase. *J Am Chem Soc* 113:6299–6300.
56. Henriksen, B.S., Anderson, J.L., Hrycyna, C.A., and Gibbs, R.A. (2005). Synthesis of desthio prenylcysteine analogs: sulfur is important for biological activity. *Bioorg Med Chem Lett* 15:5080–5083.
57. Bergman, J.A., Hahne, K., Hrycyna, C.A., and Gibbs, R.A. (2011). Lipid and sulfur substituted prenylcysteine analogs as human Icmt inhibitors. *Bioorg Med Chem Lett* 21:5616–5619.
58. Marciano, D., Benbaruch, G., Marom, M., Egozi, Y., Haklai, R., and Kloog, Y. (1995). Farnesyl derivatives of rigid carboxylic acids-inhibitors of Ras-dependent cell-growth. *J Med Chem* 38:1267–1272.
59. Marciano, D., Aharonson, Z., Varsano, T., Haklai, R., and Kloog, Y. (1997). Novel inhibitors of the prenylated protein methyltransferase reveal distinctive structural requirements. *Bioorg Med Chem Lett* 7:1709–1714.
60. Marom, M., Haklai, R., Ben-Baruch, G., Marciano, D., Egozi, Y., and Kloog, Y. (1995). Selective inhibition of Ras-dependent cell growth by farnesylthiosalicylic acid. *J Biol Chem* 270:22263–22270.
61. Winter-Vann, A.M., *et al.* (2005). A small-molecule inhibitor of isoprenylcysteine carboxyl methyltransferase with antitumor activity in cancer cells. *Proc Natl Acad Sci USA* 102:4336–4341.
62. Baron, R.A., Peterson, Y.K., Otto, J.C., Rudolph, J., and Casey, P.J. (2007). Time-dependent inhibition of isoprenylcysteine carboxyl methyltransferase by indole-based small molecules. *Biochemistry* 46:554–560.
63. Wang, M., *et al.* (2008). A small molecule inhibitor of isoprenylcysteine carboxymethyltransferase induces autophagic cell death in PC3 prostate cancer cells. *J Biol Chem* 283:18678–18684.
64. Go, M.L., Leow, J.L., Gorla, S.K., Schuller, A.P., Wang, M., and Casey, P.J. (2010). Amino derivatives of indole as potent inhibitors of isoprenylcysteine carboxyl methyltransferase. *J Med Chem* 53:6838–6850.

65. Anderson, J.L., Henriksen, B.S., Gibbs, R.A., and Hrycyna, C.A. (2005). The isoprenoid substrate specificity of isoprenylcysteine carboxylmethyltransferase—development of novel inhibitors. *J Biol Chem* 280:29454–29461.
66. Ma, Y.T., Shi, Y.Q., Lim, Y.H., McGrail, S.H., Ware, J.A., and Rando, R.R. (1994). Mechanistic studies on human platelet isoprenylated protein methyltransferase—farnesylcysteine analogs block platelet-aggregation without inhibiting the methyltransferase. *Biochemistry* 33:5414–5420.
67. Ma, Y.T., Gilbert, B.A., and Rando, R.R. (1995). Farnesylcysteine analogs to probe role of prenylated protein methyltransferase. *Methods Enzymol* 250:226–234.
68. Donelson, J.L., Hodges, H.B., MacDougall, D.D., Henriksen, B.S., Hrycyna, C.A., and Gibbs, R.A. (2006). Amide-substituted farnesylcysteine analogs as inhibitors of human isoprenylcysteine carboxyl methyltransferase. *Bioorg Med Chem Lett* 16:4420–4423.
69. Donelson, J.L., Hodges-Loaiza, H.B., Henriksen, B.S., Hrycyna, C.A., and Gibbs, R.A. (2009). Solid-phase synthesis of prenylcysteine analogs. *J Org Chem* 74:2975–2981.
70. Rostovtsev, V.V., Green, L.G., Fokin, V.V., and Sharpless, K.B. (2002). A stepwise Huisgen cycloaddition process: copper (I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew Chem Int Ed* 41:2596–2599.
71. Majmudar, J.D., Hahne, K., Hrycyna, C.A., and Gibbs, R.A. (2011). Probing the isoprenylcysteine carboxyl methyltransferase (Icmt) binding pocket: sulfonamide modified farnesyl cysteine (SMFC) analogs as Icmt inhibitors. *Bioorg Med Chem Lett* 21:2616–2620.
72. Buchanan, M.S., *et al.* (2007). Spermatinamine, the first natural product inhibitor of isoprenylcysteine carboxyl methyltransferase, a new cancer target. *Bioorg Med Chem Lett* 17:3.
73. Buchanan, M.S., *et al.* (2008). Aplysamine 6, an alkaloidal inhibitor of Isoprenylcysteine carboxyl methyltransferase from the sponge *Pseudoceratina* sp. *J Nat Prod* 71:7.
74. Garcia, J., Pereira, R., and de Lera, A.R. (2009). Total synthesis of the natural isoprenylcysteine carboxyl methyltransferase inhibitor spermatinamine. *Tetrahedron Lett* 50:5028–5030.
75. Buchanan, M.S., *et al.* (2008). Small-molecule inhibitors of the cancer target, isoprenylcysteine carboxyl methyltransferase, from *Hovea parvicalyx*. *Phytochemistry* 69:1886–1889.
76. Majmudar, J.D., *et al.* (2011). Amide-modified prenylcysteine based Icmt inhibitors: structure-activity relationships, kinetic analysis and cellular characterization. *Bioorg Med Chem.* (in press).
77. Bergman, J.A., *et al.* (2011). S-farnesyl-thiopropionic acid (FTP) triazoles as novel, potent inhibitors of isoprenylcysteine carboxyl methyltransferase (Icmt) manuscript submitted. *ACS Med Chem Lett.* (unpublished data).