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**Review Article**

# Isoprenylcysteine carboxylmethyltransferase as a target for development of novel cancer therapeutics

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**Abstract**

Isoprenylcysteine carboxylmethyltransferase (Icmt), which catalyzes the last step of the three-step post-translational modification of a group of proteins containing the so-called CaaX motif, has become the focus of research in the quest for discovering new and more effective cancer therapies. Icmt is the only enzyme that performs carboxyl methylation of isoprenoid-modified proteins, termed prenyl proteins, and hence is of crucial importance in this processing pathway. Substrates of Icmt include not only most members of the well-known Ras GTPase superfamily of proteins, but also a host of other proteins that have functional roles in important aspects of biological regulation. Efforts to assess the impact of Icmt inhibition on tumorigenesis in particular increased after protein farnesyltransferase inhibitors (FTIs) performed below expectation in solid tumors. A selective small-molecule inhibitor of Icmt termed cysmethynil has recently been described which significantly inhibits transformed cell growth and triggers cell death. The precise mechanisms by which Icmt inhibition results in antiproliferative responses, in particular which of its many substrates are involved, remain unclear and are presently being examined. Inhibitors of Icmt have the potential not only for the treatment of cancer, but also other diseases related to aberrant function of CaaX proteins, including inflammatory diseases such as rheumatoid arthritis.

## Overview of protein prenylation

Post-translational modification of protein by isoprenoid lipids, a process termed prenylation, has only been studied since the 1980s (1). In the ensuing 20 years, however, this protein modification pathway has been extensively studied, in large part due to the importance of the biological functions of many prenylated proteins (2, 3). The best-known and most extensively studied of these proteins are the members of the Ras GTPase family due to their critical involvement in many types of cancers (4).

The modification of the majority of prenylated proteins is dictated by the presence of the so-called CaaX motif at the *C*-terminus of these proteins, where the “C” designates an invariant cysteine residue, the “a” residues are generally aliphatic, and a variety of amino acids can be found at the X position (2). The identity of the X residue determines whether the protein is farnesylated or geranylgeranylated on the cysteine residue of the CaaX sequence by one of two quite specific enzymes (5). When X is serine, cysteine, alanine or glutamine, the 15-carbon farnesyl group is added to the cysteine residue by protein farnesyltransferase (FTase), whereas if the X is leucine, then the 20-carbon geranylgeranyl group is added by protein geranylgeranyltransferase type I (GGTase-I). Once prenylated, the -aaX peptide is removed on the endoplasmic reticulum membrane by a prenyl protein-specific endoprotease named Rce1 (6, 7). The exposed carboxyl group generated on prenylated cysteine by this proteolysis step is then methylated by isoprenylcysteine carboxylmethyltransferase (Icmt) (8, 9). The processing pathway for CaaX proteins is diagrammed in Figure 1.

The functional importance of the three-step modification of CaaX proteins is well established for many of these proteins, and the processing is generally important for membrane association and protein localization in the cell, as well as in protein-protein interactions (10, 11). These properties have been shown to be indispensable for many specific functions of these proteins. Recent studies have demonstrated that, although methylation as the third step is less important for hydrophobicity of the CaaX proteins, especially those that are modified by the 20-carbon geranylgeranyl isoprenoid (12, 13), the modification is nevertheless important to the function of these proteins (14-16).

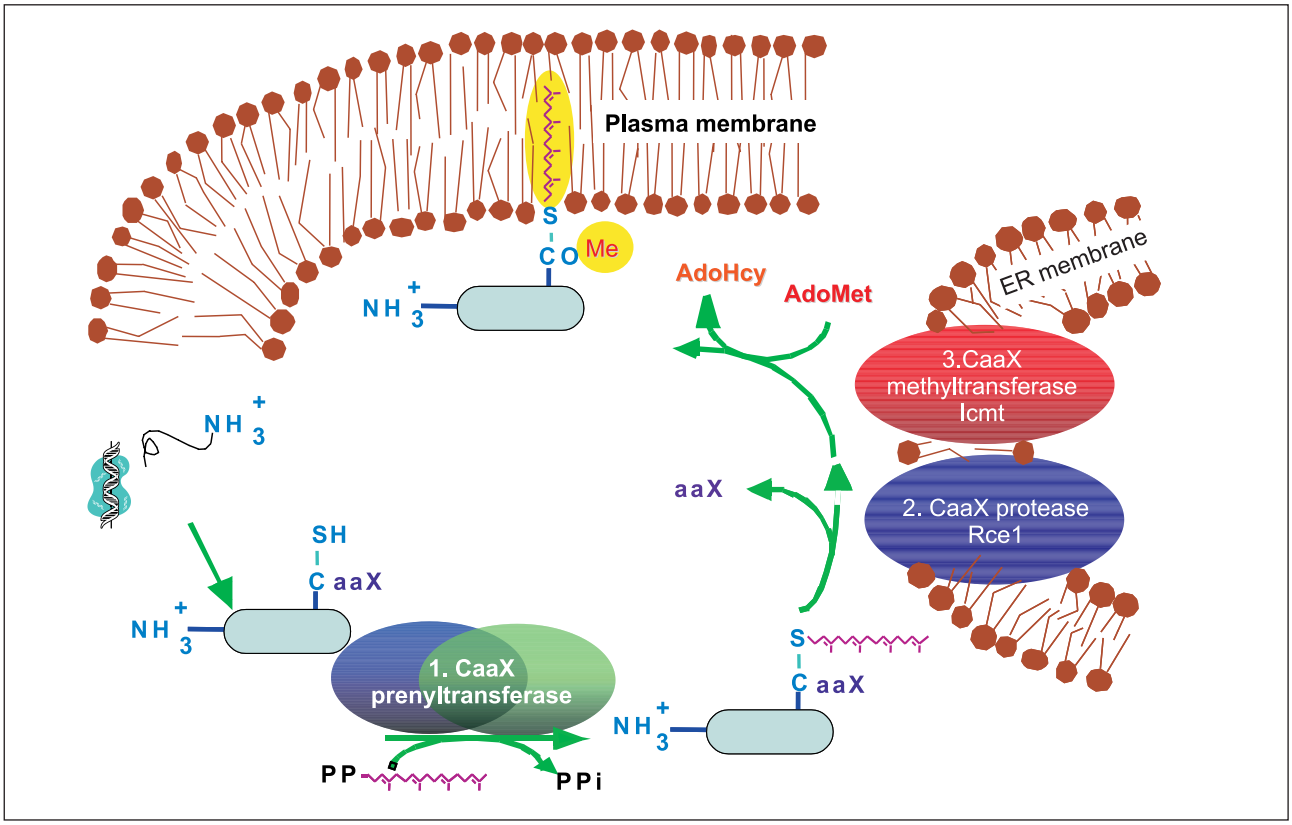


Fig. 1. Processing pathway for CaaX prenyl proteins. Upon synthesis in the cytoplasm, CaaX proteins are first modified by the appropriate CaaX prenyltransferase, either farnesyltransferase (FTase) or geranylgeranyltransferase type I (GGTase-I), after which the vast majority of CaaX proteins undergo two subsequent prenylation-dependent processing steps: proteolytic removal of the -aaX tripeptide by the CaaX protease Rce1 and carboxyl methylation of the now *C*-terminal prenylcysteine residue by isoprenylcysteine carboxylmethyltransferase (Icmt). Rce1 and Icmt are both membrane-bound proteins localized to the endoplasmic reticulum (ER). Following this 3-step modification, the modified proteins are directed to their appropriate cellular destination, which is the plasma membrane for the hypothetical protein depicted.

## Rationale for targeting protein prenylation in therapeutic strategies

Protein prenylation first came under the spotlight in the late 1980s with the recognition that a yeast mating factor, as well as yeast and mammalian Ras proteins, were modified by the 15-carbon farnesyl moiety (17, 18). These early studies also revealed that prenylation of Ras proteins was not only required for their membrane association, but also for their function, including the oncogenicity of activated forms of these proteins (19, 20). Since it was already known at that time that over 30% of human cancers exhibit elevated Ras activity, which is important for their proliferation and survival (21), the potential for targeting the prenylation pathway in the development of anticancer therapeutics became apparent. Additionally, Ras GTPases and other CaaX proteins are involved in an array of signaling pathways that are important in other pathologies dependent on aberrant proliferation, such as inflammation (4, 22).

Because the prenylation pathway is required for the function of so many important signaling proteins, there is tremendous interest, both in academia and in industry, in developing inhibitors of this pathway in the hope of providing novel therapeutic strategies for fighting diseases. The most advanced of these efforts are those targeting FTase, where pharmaceutical companies have invested considerable resources toward finding and evaluating specific inhibitors, termed FTIs (23, 24). Hundreds of patents have been issued covering a broad array of FTIs in the past 10 years (25), and several lead compounds have shown impressive anticancer activity *in vitro* and in mouse tumor models (24). However, these agents have not fared as well in clinical trials, particularly as single agents against solid tumors. While there is still considerable effort dedicated toward evaluating FTIs in solid tumors, for example in combination with cytotoxic or other drugs (26), much of the attention in applying FTIs in cancer therapy has shifted to hematological cancers, where impressive preclinical and phase II activity has been seen for the compounds (27).

Just why FTIs have not performed up to expectation in solid tumors, and why many types of cancer, even those with activated *RAS* oncogenes, fail to respond to FTIs, has been a subject of much debate in the oncology community (26, 28-30). Much of this variability is undoubt-

edly due to the multifactorial nature of human cancers, but a significant amount of attention has also been placed on the process termed “alternative prenylation”, whereby CaaX proteins, including some Ras isoforms, can be modified by GGTase-I when FTase is inhibited (31, 32). The discovery of alternative prenylation sparked interest not only in inhibitors of GGTase-I, but also in finding compounds that block activated forms of both farnesylated and geranylgeranylated CaaX proteins through inhibition of the distal processing steps in the pathway, *i.e*., Rce1 or Icmt, since there is but a single enzyme for both of these steps that processes both farnesylated and geranylgeranylated proteins (33, 34).

There are many proteins in addition to Ras that go through the CaaX sequence-targeted three-step modification which play important roles in essential cellular functions. A structural bioinformatics analysis of the human genome, using information derived from the analysis of CaaX recognition by FTase and GGTase-I, indicates roughly 120 human proteins that would be predicted to be processed via this pathway (35). Among these proteins are the canonical Ras proteins and their close relatives, the Rho GTPases, the $\gamma$ subunits of heterotrimeric G-proteins, several protein kinases and phosphatases, nuclear lamins A and B, and a number of other proteins involved in important cell functions. The majority of these CaaX proteins are involved in signal transduction and/or biological regulatory events. Of these probable CaaX prenylated proteins, their *C*-terminal residues predict that a slight majority will be geranylgeranylated, highlighting the need for analysis of the biological consequences of GGTase-I, Rce1 and Icmt inhibitors, as well as FTIs.

The first genetic targeting of postprenylation processing in mammalian systems was performed on Rce1 (36). In the model systems examined to date, the effects of Rce1 deletion on oncogenesis have been rather modest. While deletion of Rce1 in fibroblasts did reduce Ras-induced transformation (37), the effects were much less than those observed with FTI treatment. However, one promising finding from this study in terms of targeting Rce1 in cancer therapy was that elimination of the enzyme rendered tumor cells much more sensitive to FTI treatment. Hence, pharmacological targeting of Rce1 might be an approach to enhancing the efficacy of FTI in clinical settings.

In contrast to Rce1 mouse knockouts, much more promising results have been obtained in genetic targeting of Icmt in that deletion of Icmt almost completely blocked transformation of fibroblasts by oncogenic K-Ras (a form which is subject to the alternate prenylation noted above), and surprisingly by activated B-Raf as well (16). These findings, and those noted below on pharmacological targeting of Icmt, have spurred interest in this enzyme as an anticancer target.

## Properties of Icmt

The first evidence for *C*-terminal methylation of CaaX proteins came from studies of the a-type mating factor of budding yeast (18). Methylation occurring at the *C*-terminal-modified cysteine residues of other CaaX proteins, such as Ras and lamin B protein, was also detected about the same time (1). Also during this period, studies of mating in yeast led to the identification of several mutants that were defective in this *C*-terminal processing. One of these genes, *RAM1* or *DPR1*, was found to encode a subunit of the yeast FTase (38). Mutants of another gene identified in this screen, *STE14*, were found to have a defect in the production of active a-factor due to an inability to carry out the *C*-terminal methylation of a-factor such that, in *STE14* mutants, the biological activities of a-factor are dramatically reduced when it is not methylated, which renders the strain sterile (9, 39).

Mammalian Icmt was first identified through a bioinformatics approach using sequence information that emerged from the aforementioned cloning of *STE14*, as well as its orthologues in *Schizosaccharomyces pombe* and *Xenopus laevis* (8, 40). Hydrophobicity analysis of the primary sequence of human Icmt suggests that it contains six putative membrane-spanning domains. Using polyclonal antiserum raised against a conserved region of the *N*-terminus sequence, a 33-kDa protein from HL-60 and COS cells was detected on Western blot. Through the use of this antiserum, endogenous Icmt was localized to the endoplasmic reticulum (ER) membrane in mammalian cells (8). Since the protein prenyltransferases are cytosolic enzymes, this localization, and the ER localization reported for the Rce1 protease (41), presumably reflects that the CaaX protein substrates of Icmt are prenylated in the cytosol as free proteins and then channeled to the ER membrane for proteolysis and methylation before distribution to final destinations. The final location of the modified proteins could be the cytoplasmic membrane surfaces, in the case of Ras GTPases and the $\gamma$ subunits of heterotrimeric G-proteins, in the cytoplasm in a complex with other proteins that shield the hydrophobicity of the isoprenoid for many Rho GTPases, or the nucleus with nuclear lamins.

Biochemical analysis of Icmt has been hampered by its properties as an integral membrane protein. This has been especially true for the mammalian enzyme, but less so for *Saccharomyces cerevisiae* Icmt, which has been effectively solubilized, purified and reconstituted into liposomes, allowing initial characterization of this enzyme in purified form (42). In spite of difficulties with this approach with the mammalian enzyme, detailed kinetic analyses to establish the steady-state kinetic mechanism of the enzyme have been carried out via analysis of both endogenous activity in cell membranes (43) and recombinant human Icmt expressed in Sf9 cells (44).

Analysis of Icmt knockout mice revealed that these mice demised at the mid-gestational stage, demonstrating the importance of Icmt activity in early development (45). No specific defects were observed that could account for the lethality of the disruption, but a subsequent study demonstrated that Icmt function is important in the early stages of liver development in mice (46). In the latter study, the authors speculated that Icmt impacts

on fibroblast growth factor (FGF) signaling by modulating Ras during hepatocyte specification. The study of Icmt-deficient mice has begun to elucidate the important role of Icmt in mouse development and to provide attractive models for evaluating the consequences of limiting Icmt activity on physiological and pathological processes.

## Icmt as a target for drug discovery

While there has been increasing interest in targeting Icmt in cancer therapy, as early as 1991 the farnesylcysteine analogue *N*-acetyl-*S*-farnesyl-L-cysteine (AFC; Fig. 2) was studied as an inhibitor of carboxyl methylation of CaaX proteins (47). In this study, AFC was reported to inhibit methylation of Ras in transformed fibroblast cells, although it had little effect on the rates of proliferation of these cells. Another prenylcysteine analogue that has been studied as an Icmt inhibitor is farnesylthiosalisylic acid (FTS; Fig. 2). In a cell-free system, FTS was found to inhibit the methylation of GTP-binding proteins such as Ras (48). FTS treatment interferes with the growth of human H-Ras-transformed Rat1 cells, but has only minimal cytotoxicity in a variety of other transformed cell lines (49). One conclusion from these studies was that not all prenylated proteins are equally affected by the treatment of cells with the inhibitor; Ras proteins in particular were seen to be more affected by FTS treatment than some other CaaX proteins. This finding led to the conclusion that the cellular activity of FTS was more likely due to its ability to dislodge Ras from membranes rather than its ability to inhibit CaaX protein methylation (49, 50).

In spite of the early reported successes of targeting Icmt with small-molecule substrate mimics such as AFC, a number of studies in the 1990s, in addition to those noted above with FTS, cast doubt on whether the observed biological effects of treating cells with prenylcysteine analogues were actually due to Icmt inhibition. For example, the ability of AFC to inhibit macrophage chemotaxis was later linked to the direct blockade of the interaction between receptor and G-proteins (the latter of which, of course, contain a CaaX protein subunit) rather than inhibition of carboxyl methylation (51). Additional studies have made it clear that compounds such as AFC have significant effects on cells via an impact on processes that do not involve inhibition of Icmt (52, 53). The most likely molecular targets responsible for pharmacological consequences of these compounds are protein-protein interactions in which prenylated proteins participate; as structural mimics of the carboxy-terminal prenylcysteine of processed CaaX proteins, these compounds likely compete for prenylation-dependent protein-protein interactions. Hence, AFC and related compounds are probably best viewed as inhibitors of prenylcysteine-dependent interactions, which can include substrate recognition by Icmt, but this may be only a minor component of their pharmacology (34). Whether this property extends to a newer generation of prenylcysteine-based inhibitors of Icmt (54) is unclear. In any event, this series of studies highlights a general problem in designing enzyme inhibitors based upon a substrate structure that is also represented in a variety of cellular macromolecules.

A mechanistically distinct approach to targeting Icmt has taken advantage of the fact that, as a methyltransferase, it is likely subject to inhibition by one of its reaction products, *S*-adenosylhomocysteine (AdoHcy). Using a variety of methods to bring about increased levels of

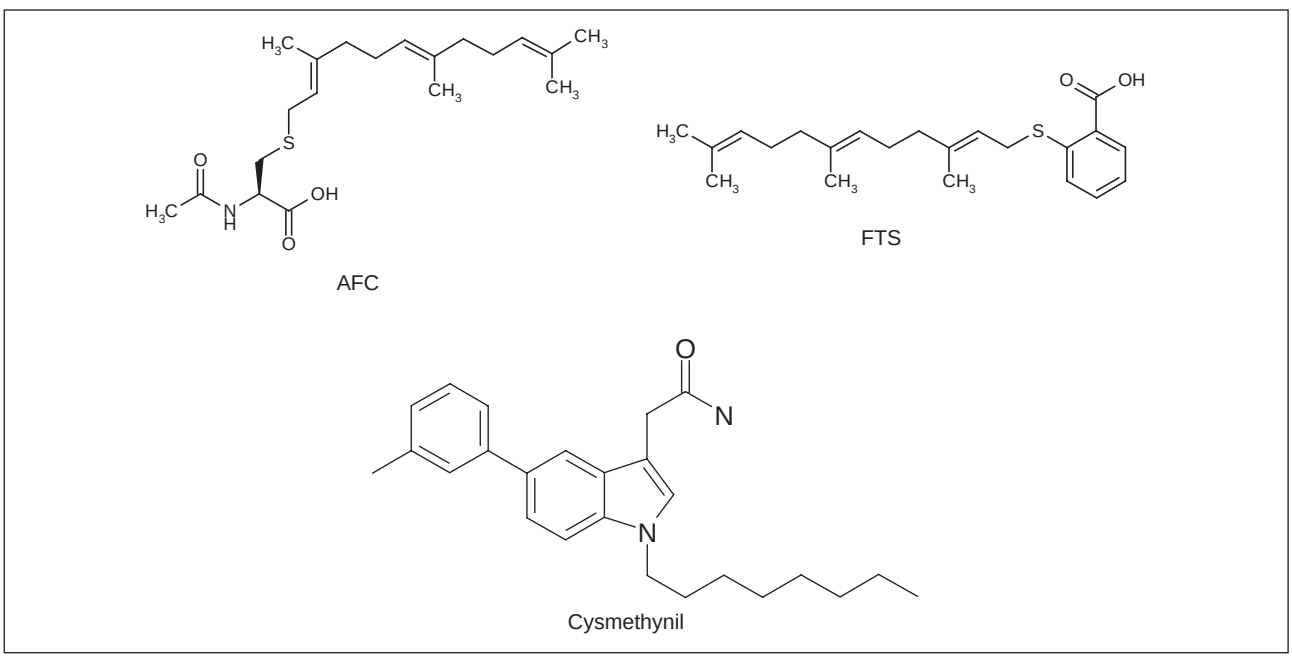


Fig. 2. Structures of representative isoprenylcysteine carboxylmethyltransferase (Icmt) inhibitors. Shown are the structures of two well-characterized prenylcysteine analogues, AFC and FTS, and the novel small-molecule inhibitor cysmethynil.

AdoHcy in cells, the resulting inhibition of Icmt has been linked to reduced proliferation in a number of cell types (55), and an increase in apoptosis in endothelial cells in particular (56, 57). In one such study from our lab, the classic anticancer therapeutic methotrexate was employed to elevate AdoHcy in cancer cells (58). Via its impact on 1-carbon metabolism in cells, methotrexate treatment leads to increased levels of homocysteine and a resultant increase in AdoHcy. In a colon cell line treated with methotrexate, Ras methylation was profoundly inhibited and this hypomethylation was accompanied by mis localization and a significant decrease in Ras signaling. Although AdoHcy is not a selective inhibitor of Icmt, but rather a general inhibitor of cellular methyltransferases (59), these studies nonetheless supported the hypothesis that targeting Icmt might inhibit the proliferation of cancer cells and enhance their apoptotic death.

In an attempt to move beyond substrate- and product-based inhibitors of Icmt and discover a pharmacological agent with higher potency and selectivity, we undertook the screening of a diverse chemical library for novel Icmt inhibitors. This effort led to the identification of a selective small-molecule inhibitor of Icmt, cysmethynil (Fig. 2) (15). Treatment of human colon cancer cells with cysmethynil generated many of the same cellular characteristics noted above with the Icmt knockout models, including Ras mislocalization, impaired Ras signaling and inhibition of oncogenesis as assessed by tumor cell growth in soft agar (Fig. 3). Moreover, these effects were reversible upon overexpression of Icmt, indicating mechanism-based activity of the compound. Also, cysmethynil treatment did not impair the proper localization of membrane protein modified by myristoyl/palmitoyl, demonstrating that the compound does not cause a global disruption of protein trafficking to the cell membrane. The identification of cysmethynil as an Icmt inhibitor has provided a selective pharmacological tool to probe the potential functional consequences of CaaX protein methylation in cellular systems and also the involvement of Icmt in both normal and pathological cellular processes.

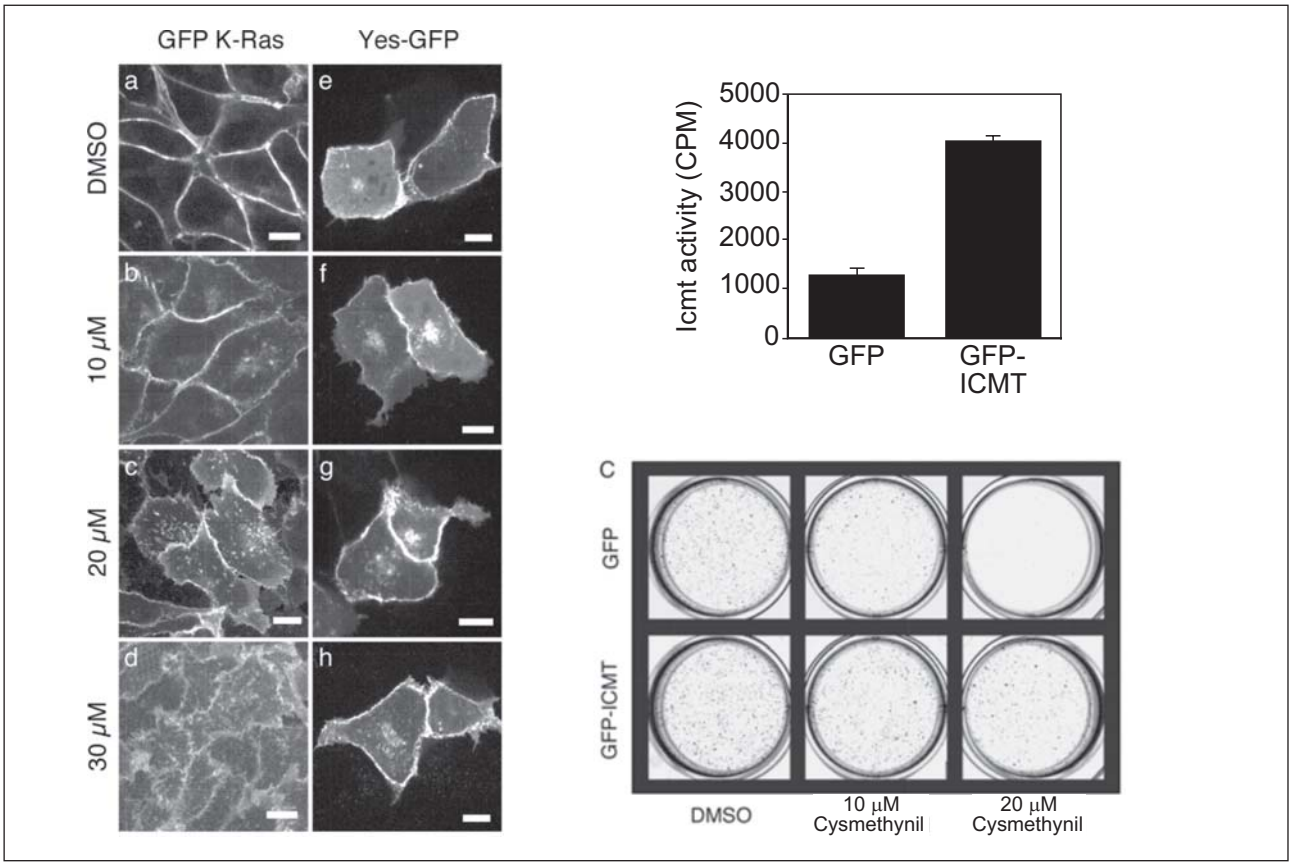


Fig. 3. Impact of cysmethynil inhibition of isoprenylcysteine carboxylmethyltransferase (Icmt) on Ras localization and oncogenic transformation. *Left panel*. Mislocalization of GFP-Ras in cysmethynil-treated cells. MDCK cells expressing GFP-tagged K-Ras or the *N*-myristoylated protein Yes-GFP were treated with DMSO or the indicated concentration of cysmethynil prior to imaging by confocal microscopy. *Upper right panel*. Creation of cell lines stably overexpressing Icmt. DKOB8 colon cancer cells were engineered to stably express either GFP alone or a GFP-Icmt fusion protein. Membrane fractions from GFP- and GFP-ICMT-expressing lines were assayed for Icmt activity. *Lower right panel*. Impact of cysmethynil on anchorage-independent growth of DKOB8 colon cancer cells. DKOB8 cells stably expressing GFP or GFP-ICMT, as indicated, were suspended in soft agar that contained either DMSO or 10 or 20 μM cysmethynil, as indicated. After 3 weeks of growth, colonies were stained and imaged. Cysmethynil treatment dramatically inhibited the growth of the cancer cells in soft agar, and the effect was markedly diminished in cells overexpressing Icmt. Reproduced with permission from Ref. 15.

## Potential mechanisms for biological effects of Icmt inhibition

Even though both *in vitro* and *in vivo* data have provided a strong argument for the therapeutic potential of targeting Icmt, the molecular mechanisms that contribute to the biological effects of Icmt inhibitors are far from clear. Contrary to the initial belief that the effects of inhibition of CaaX protein processing might be primarily due to an impact on Ras signaling, growing evidence points to a much more complex picture. As noted above, it is now appreciated that more than 100 proteins in human cells undergo CaaX motif processing, and many of these are involved in key regulatory functions. The Ras superfamily of GTPases alone contains more than 50 members. These proteins are categorized into six subfamilies based on their protein sequences: Ras, Rho, Arf, Rab, Ran and Sar; of these, the members of the Ras and Rho subfamilies are CaaX proteins, and the Rab proteins are also prenylated, but via a somewhat distinct process than CaaX proteins. Although not CaaX proteins, a subset of Rab proteins are subject to *C*-terminal methylation by Icmt (45, 60).

Each subfamily of the Ras superfamily performs distinct functions, although there may be some overlap. The roles of the Ras subfamily in signaling pathways have been well established (4), and the Rho subfamily is involved in cytoskeletal events, cell proliferation, survival and migration pathways (61). Moreover, even when one considers just the canonical members of the Ras subfamily, *i.e.*, H-, K- and N-Ras, one finds an array of signaling pathways under their control; the three most widely studied are the Raf/MEK (mitogen-activated protein kinase/ERK kinase)/ERK (extracellular signal-regulated kinase) pathway, the PI3-kinase (phosphatidylinositol 3-kinase)/Akt pathway and the RalGDS (Ral guanine nucleotide dissociation stimulator) pathway, but many more effectors of Ras have been identified (4). This multitude of pathways and potential crosstalk among them and with others form a complex and delicate controlling system for cell growth, differentiation, survival and apoptosis, and CaaX proteins participate in one way or another in most, if not all, of the pathways.

In one of the first studies to look beyond Ras signaling as a primary determinant of the consequences of Icmt inhibition, Bergo and colleagues followed up on their surprising finding that although Icmt knockout in mouse cells resulted in 90-95% of Ras protein being mislocalized, there was no discernable effect on either ERK or Akt1 activation following growth factor stimulation (62). In the follow-up study (16), these researchers looked for another signaling component to explain the growth- and transformation-delaying effects of Icmt inhibition. In this regard, RhoA was singled out as a potential player, given its known involvement in oncogenesis. Indeed, inactivation of Icmt led to a marked reduction in steady-state levels of RhoA in cells via a process that was found to be due to destabilization of the protein, as its half-life was much shorter in the Icmt-null cells (16). Since a previous study had provided evidence that concomitant activation of Ras and reduction of Rho resulted in the upregulation of $p21^{WAF1/CIP1}$, leading to cell cycle arrest (63), the Icmt-null cells were examined and indeed found to exhibit an increased level of $p21^{CIP1}$. When experiments were performed in the cell lacking $p21^{CIP1}$, inactivation of Icmt had no measurable effect on tumorigenesis, indicating the importance of $p21^{CIP1}$, and also by extension of reduced Rho levels, in the antitumor response to Icmt inactivation. How broadly applicable to other cell types these results will be is not yet clear, but what is clear is that a Ras-centric view of these compounds is overly naïve and considerable work is still required to decipher the mechanisms behind the cellular effects seen with Icmt inhibition. As Icmt has many substrates that are involved in important cellular functions, it is very likely that Icmt inhibition exerts cellular effects through a variety of mechanisms warranting further investigation.

## Potential application of Icmt inhibitors to diseases other than cancer

The majority of studies on Icmt inhibition so far have focused on the therapeutic potential of this approach in fighting cancer. There are, however, some studies on the implications of Icmt inhibition to other illnesses. For example, the involvement of Rac proteins in superoxide release and the importance of this process in the pathogenesis of rheumatoid arthritis (64) suggest that Icmt inhibitors may impact on this disease process. Dysregulation of the Rho pathway has been implicated in multiple pathological conditions in addition to cancer, including cardiovascular and hepatic diseases. Therefore, the interest in targeting these proteins has increased. Rho activity has a clear function in angiogenesis (65), which in turn plays a critical role in rheumatoid arthritis, atherosclerosis, psoriasis and diabetic retinopathy, as well as in the pathogenesis of tumors (66). In multiple sclerosis, infiltration of leukocytes and monocytes into the central nervous system is part of an inappropriate inflammatory response, leading to demyelination of axons and nerve damage which presents as motor and cognitive impairment. Recent evidence suggests that this inflammatory cell migration requires Rho signaling and inhibition of prenylation can lead to decreased disease progression (67, 68). Additionally, the replication of hepatitis C virus (HCV) in liver cells requires a CaaX protein termed Fbl2, and evidence indicates that HCV replication can be attenuated by inhibiting prenylation of this protein (69, 70). While the methylation status of Fbl2 has not yet been examined, it is intriguing to speculate that inhibition of Icmt might provide a potential new therapeutic approach for this increasingly prevalent disease (69, 70).

## Summary and conclusions

If the last 15 years was the era of targeting farnesylation as an approach to blocking CaaX proteins, specifically Ras function in cancer therapeutics, the next 15 years will likely be the era when the precise contributions

of impairing activities of different CaaX proteins on a variety of biological and pathobiological processes will be unraveled. It is unlikely that the biological effects of an inhibitor of the prenylation pathway will be traced to effects on a single CaaX protein substrate, but rather to a number of such substrates, which may include Ras proteins, but also a suite of others depending on the biological process one is examining. Furthermore, Icmt inhibition will likely take a center stage in these efforts due to its impact on both farnesylated and geranylgeranylated CaaX proteins and the consequences of its inhibition in cells.

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