Time-Dependent Inhibition of Isoprenylcysteine Carboxyl Methyltransferase by Indole-Based Small Molecules[†]

Rudi A. Baron,[‡,§] Yuri K. Peterson,[‡] James C. Otto,[‡] Johannes Rudolph,[||] and Patrick J. Casey*[,‡,||]

Department of Pharmacology and Cancer Biology and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT: Isoprenylcysteine carboxyl methyltransferase (Icmt) catalyzes the methylation of the C-terminal prenylcysteine found on prenylated proteins. Numerous studies have shown that the methylation step is important for the correct localization and function of many prenylated proteins, most notably GTPases in the Ras superfamily. We recently reported identification of a small molecule derived from an indole core as a potent, cell-active inhibitor of Icmt whose potency was increased upon preincubation with the enzyme [Winter-Vann, A. M., Baron, R. A., et al. (2005) *Proc. Natl. Acad. Sci. U.S.A. 102* (12), 4336−41]. In the study presented here, we performed a kinetic characterization of this time-dependent inhibition of Icmt by 2-[5-(3-methylphenyl)-1-octyl-1*H*-indol-3-yl]acetamide (cysmethynil). These analyses revealed that cysmethynil is a competitive inhibitor with respect to the isoprenylated cysteine substrate and a noncompetitive inhibitor with respect to AdoMet, the methyl donor in the reaction. The K_i of cysmethynil for Icmt, which represents the dissociation constant of the initial complex with the enzyme, was 2.39 ± 0.02 μM, and the K_i^*, which is the overall dissociation constant of the inhibitor for the final complex, was 0.14 ± 0.01 μM. The first-order rate constant for the conversion of the initial enzyme−inhibitor complex to the final high-affinity complex was 0.87 ± 0.06 min^{-1}, and that for the reverse process was 0.053 ± 0.003 min^{-1}; the latter rate constant corresponds to a half-life for the high-affinity complex of 15 min. Structure−activity relationships of a number of closely related indole compounds revealed that the hydrophobicity of the substituent on the nitrogen of the indole core was responsible for the manifestation of time-dependent inhibition. These findings markedly enhance our understanding of the mechanism of inhibition of Icmt by this indole class of compounds and should facilitate ongoing efforts to assess the potential of targeting this enzyme in anticancer drug design.

Many key regulatory proteins in eukaryotic cells are modified at their C-terminus via a process initiated by covalent attachment of 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoids to a conserved cysteine residue. This processing, termed protein prenylation, is critical for the correct localization and function of these proteins (*1*, *2*). The majority of prenylated proteins contain a C-terminal CaaX motif, where C denotes the Cys residue that is modified, the a's are generally aliphatic residues, and X can be any of a number of amino acids. Specific recognition of the CaaX motif by protein farnesyltransferase (FTase)[1] or protein geranylgeranyltransferase type I (GGTase-I) is highly dependent on the X residue (*3*), and the prenylation of the Cys is followed by proteolytic removal of the aaX residues by the endoprotease Rce1 (*4*, *5*) and methylation of the newly exposed prenylcysteine by the enzyme isoprenylcysteine carboxyl methyltransferase (Icmt) (*6*). These final two processing steps appear to further increase the general hydrophobicity of the proteins and also provide a unique determinant for protein−protein interactions (*7*, *8*).

Prenylation of the CaaX proteins has attracted considerable interest, in large part because of the key role of the activated forms of Ras in the pathogenesis of human cancers (*9*). Prenylation is required for the proper localization and function of Ras proteins (*1*, *3*), and the growth of Ras-dependent tumors in mice can be inhibited by pharmacological blockade of FTase (*10*−*12*). However, K-Ras and N-Ras can largely escape this inhibition due to a process termed cross-prenylation whereby, when FTase is inhibited, GGTase-I can modify the proteins at an appreciable rate (*13*, *14*). In contrast to the protein prenyltransferases, however, there appear to be only one protease and one methyltrans-

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* To whom correspondence should be addressed: Box 3813, Duke University Medical Center, Durham, NC 27710-3813. E-mail: casey006@mc.duke.edu. Phone: (919) 613-8613. Fax: (919) 613-8642.

[‡] Department of Pharmacology and Cancer Biology.

[§] Present address: Division of Biomedical Sciences, Imperial College London, Sir Alexander Fleming Building, South Kensington, London SW7 2AZ, U.K.

[||] Department of Biochemistry.

[1] Abbreviations: Icmt, isoprenylcysteine carboxyl methyltransferase; FTase, protein farnesyltransferase; FPP, farnesyl diphosphate; GGTase, protein geranylgeranyltransferase; CaaX, conserved carboxyl-terminal motif of FTase substrates; FK-Ras, farnesylated K-Ras protein; FK-RasCOO$^-$, farnesylated and Rce1-proteolyzed K-Ras protein; cysmethynil, 2-[5-(3-methylphenyl)-1-octyl-1*H*-indol-3-yl]acetamide; AFC, *N*-acetyl-*S*-farnesyl-L-cysteine; AGGC, *N*-acetyl-*S*-geranylgeranyl-L-cysteine; FTA, *S*-farnesylthioacetic acid; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosylhomocysteine; AFCMe, *N*-acetyl-*S*-farnesyl-L-cysteine methyl ester; BFC, biotin-*S*-farnesyl-L-cysteine.

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ferase that each handle both farnesylated and geranylgeranylated proteins (*15*, *16*). Thus, the blockade of one of these steps would be expected to impact all Ras isoforms. Indeed, recent studies have shown that genetic disruption of Icmt in cells dramatically attenuates their ability to be transformed by oncogenic K-Ras (*17*), and treatment of a human colon cancer cell line with a specific Icmt inhibitor blocks anchorage-independent growth of the cancer cells (*18*).

We recently reported the identification of a potent and selective small molecule inhibitor from a screen of a diverse chemical library (*18*). Treatment of cancer cells with this compound, termed cysmethynil, impaired Ras localization and function while reversing the oncogenic phenotype of the cells. Preincubation of Icmt with cysmethynil decreased the IC_{50} for inhibition, suggesting that the compound was a time-dependent inhibitor of the enzyme (*18*). In the study presented here, we establish that cysmethynil does indeed act as a classical time-dependent inhibitor of Icmt and elucidate the kinetics of this process. Furthermore, structure−activity relationships of a series of related molecules revealed a critical determinant for the ability of this class of compounds to exhibit time-dependent inhibition.

EXPERIMENTAL PROCEDURES

Materials. Sf9 membranes containing recombinant Rce1 or Icmt, termed Rce1 membranes or Icmt membranes, respectively, were prepared as described previously (*15*, *19*). Farnesylated and Rce1-proteolyzed K-Ras (FK-RasCOO$^-$) was made by in vitro modification of bacterially expressed K-Ras with purified FTase and recombinant Rce1 as described previously (*20*). Farnesyl pyrophosphate (FPP) was purchased from Biomol, AdoMet from Sigma-Aldrich, [^{3}H-*methyl*]AdoMet from Perkin-Elmer, and AdoHcy from Fluka. FTA and AFC were made in house. Cysmethynil was prepared by the Duke Small Molecule Synthesis Facility as described previously (*18*) and purity assessed by mass spectrometry and NMR. All other indole-based compounds used in these studies were selected from the SAR10K chemical library obtained from PPD Discovery (Research Triangle Park, NC).

Measurement of Icmt Activity. Icmt activity was determined by following incorporation of ^{3}H from [^{3}H]AdoMet into the Ras protein substrate that had been subjected to farnesylation and proteolytic removal of the three C-terminal residues (FK-RasCOO$^-$). Briefly, the standard reaction mixture contained FK-RasCOO$^-$ (2 μM) and [^{3}H]AdoMet (5 μM, 1.3 Ci/mmol) in 100 mM Hepes buffer (pH 7.4) and 5 mM $MgCl_2$ in a total volume of 50 μL; reactions were initiated by addition of recombinant Icmt (0.2 μg of Icmt membranes) and conducted for varying times at 37 °C. Reactions were quenched by addition of 500 μL of 2% SDS and bovine brain cytosol (50 μg, to increase the precipitation efficiency), followed by 500 μL of 30% trichloroacetic acid to precipitate proteins. Precipitated proteins were recovered on glass fiber filters by vacuum filtration; the filters were washed three times with 2 mL of 6% trichloroacetic acid and dried, and the radioactivity was determined by scintillation counting. Alterations to this standard protocol, e.g., variation of substrate concentration, addition of inhibitory compound, or preincubation of enzyme with inhibitor prior to initiation of the reaction with substrates, are noted in the respective figure legends.

Evaluation of the onset of high-affinity inhibition of Icmt by cysmethynil was performed by adding enzyme to the complete reaction mixture noted above (although scaled up in volume) in the presence of specified inhibitor concentrations. At various time intervals, 50 μL aliquots of the reaction mixture were removed and added to the SDS quench solution noted above, and the product protein was harvested and radioactivity determined. In the absence of added inhibitor, time courses were linear for at least 20 min.

Evaluation of cysmethynil reversibility was performed by dilution analysis using an assay employing biotin-*S*-farnesyl-L-cysteine (BFC) as the prenylcysteine substrate (*20*). Sf9 membranes (0.5 μg/μL) overexpressing human Icmt were preincubated either with vehicle or with the specified concentrations of cysmethynil in buffer [70 mM HEPES (pH 7.4) and 100 mM NaCl] at 37 °C for 30 min. Samples were then diluted 50× into buffer containing just the two substrates [10 μM BFC and 5 μM [^{3}H]AdoMet (7.5 Ci/mmol)] alone (for conditionss designated 0-0 and 50-1, respectively) or into the same buffer with the substrates and additional cysmethynil [either 1 μM (for the condition designated 1-1) or 50 μM (for the condition designated 50-50)]. To avoid the complex kinetics of the time-dependent restoration of activity in the diluted sample, product formation was monitored at time points greater than two half-lives of the EI* complex. Samples (50 μL) were withdrawn at times indicated in the Figure 2D and reactions quenched, and the product was quantitated as described above.

Kinetic Analyses. For the inhibition pattern experiments with cysmethynil, data were collected for times prior to EI* formation (see below), and thus, the extent of product accumulation was linear with respect to time. The observed initial velocity data were fitted to

$$v_o = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]} \quad (1)$$

for competitive inhibition or

$$v_o = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S](1 + [I]/K_i)} \quad (2)$$

for noncompetitive inhibition, where K_m is the Michaelis constant for the varied substrate S and K_i is either the competitive or noncompetitive inhibition constant for cysmethynil. The K_i and associated SEM for cysmethynil were calculated using shared parameter curve fitting for all inhibitor concentrations using the average of duplicate determinations. The error represents the divergence between fitted curves. The kinetic analysis of the time-dependent inhibition of cysmethynil was performed according to the method of Morrison and Walsh (*21*). For a slow, tight-binding inhibitor where the inhibitor concentration exceeds the enzyme concentration, progress curves are biphasic, exhibiting an initial burst of activity, followed by a slower, steady-state rate. This type of inhibition is described by

$$P = v_s t + (v_o - v_s)[1 - \exp(-k_{obs}t)]/k_{obs} \quad (3)$$

where P is the concentration of product formed at any time t, v_o is the initial velocity, v_s is the final steady-state velocity, and k_{obs} is the apparent first-order rate constant for the

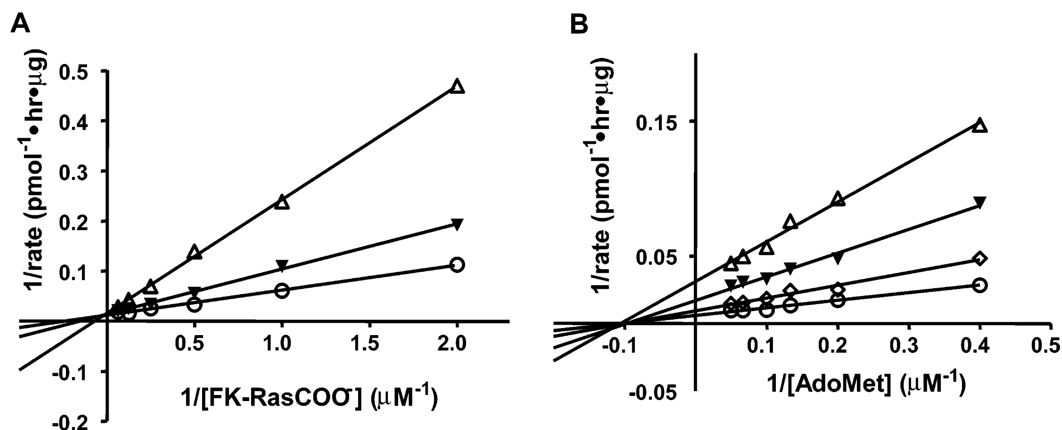

FIGURE 1: Characterization of the pattern of inhibition of Icmt by cysmethynil. (A) Lineweaver−Burk plot for the inhibition of Icmt by cysmethynil when FK-RasCOO$^-$ was the varied substrate. Icmt assays (see Experimental Procedures) were carried out in the presence of a fixed concentration of AdoMet substrate (5 μM) and the indicated concentrations of FK-RasCOO$^-$ at 0 (○), 4 (▼), or 10 μM (△) cysmethynil. Data were fitted to eq 1. (B) Lineweaver−Burk plot for the inhibition of FK-RasCOO$^-$ methylation when AdoMet was the varied substrate. Icmt assays (see above) were carried out in the presence of a fixed concentration of FK-RasCOO$^-$ substrate (2 μM) and the indicated concentrations of AdoMet at 0 (○), 1 (◇), 4 (▼), or 10 μM (△) cysmethynil. Data were fitted to eq 2. For both panels, data represent the means of duplicate determinations from a single experiment that is representative of three such experiments.

conversion from v_o to v_s. The rate constants k_3 and k_4 were obtained by fitting to

$$k_{obs} = k_4 \frac{1 + \frac{[I]}{K_i^*(1 + [S]/K_m)}}{1 + \frac{[I]}{K_i(1 + [S]/K_m)}} \quad (4)$$

$$k_{obs} = k_4 + k_3\left(\frac{[I]/K_i}{1 + [S]/K_m + [I]/K_i}\right) \quad (5)$$

where K_i^* is the overall inhibition constant accounting for both the initial binding and the slow conversion to a tight-binding complex. All equation fitting was performed using nonlinear regression in Microsoft Excel and GraphPad Prism.

RESULTS

General Pattern of Inhibition of Icmt by Cysmethynil. Inhibition of Icmt by cysmethynil {2-[5-(3-methylphenyl)-1-octyl-1*H*-indol-3-yl]acetamide} displayed high specificity for this enzyme over other enzymes in the prenylation pathway and other unrelated methyltransferases (*18*); these findings suggested that it would not generally target either the isoprenoid or AdoMet binding sites on the enzyme. Hence, our first series of kinetic experiments with this compound were designed to classify the inhibition pattern with respect to substrates. Equations describing competitive, noncompetitive, and uncompetitive inhibition were fit to the data for FK-RasCOO$^-$ in Figure 1. Visual inspection of the graphs and analysis of parameters, including K_m, V_{max}, and variance, indicate the data are best described by competitive inhibition. A double-reciprocal presentation of the data obtained from this series of experiments is shown in Figure 1 and clearly indicates that cysmethynil is a competitive inhibitor with respect to the farnesylated protein substrate (Figure 1A); the calculated K_i under the conditions employed in this assay was 2.2 ± 0.5 μM. The K_m determined for K-Ras under these conditions was 2.9 μM, and the V_{max} was 65.8 pmol/h. When the AdoMet concentration was varied at fixed FK-RasCOO$^-$ and a number of fixed cysmethynil concentrations, the lines intercept at a negative value on the *x*-axis, indicating that inhibition by the compound is noncompetitive with respect to AdoMet (Figure 1B); the K_i value determined under these conditions was 1.9 ± 0.2 μM, and the K_m for AdoMet under these conditions was 7.1 μM. As the kinetic mechanism for Icmt is ordered with AdoMet binding first (*20*, *22*), this inhibition pattern indicates that the inhibitor associates with either the free enzyme or the AdoMet−Icmt complex.

Analysis of Time-Dependent Inhibition of Icmt by Cysmethynil. As noted in the introductory section, in our initial characterization of cysmethynil inhibition of Icmt, a decrease in the IC_{50} when the compound was preincubated with enzyme prior to addition of substrates was noted (*18*). This finding prompted us to closely examine the impact of preincubating the enzyme with the compound. Analysis of the rate of product formation versus the time of preincubation with cysmethynil did indeed reveal concave-downward behavior, with increasing curvature at higher inhibitor concentrations (Figure 2A). Such nonlinear rates of product formation, coupled with a decrease in the apparent IC_{50} values under conditions in which inhibitor is preincubated with enzyme, strongly suggested that cysmethynil could be classified as a slow-binding inhibitor of Icmt (*21*). We also examined the rates of product formation in the presence of other known inhibitors of the enzyme that function as substrate or product mimics, namely, AdoHCy (*23*), farnesylthioacetic acid (FTA) (*24*), and AFC (*25*). None of these compounds demonstrated evidence of time-dependent inhibition of the enzyme (Figure 2B).

We also assessed whether the time-dependent reduction of enzyme activity by cysmethynil was reversible or irreversible. The time course of product formation was measured in presence of 2 μM cysmethynil alone or when the competing substrate, FK-RasCOO$^-$, was added in excess 10 min into the progress curve. The data, shown in Figure 2C, revealed that product formation could be recovered by the addition of excess substrate, suggesting reversible inhibition. To further investigate and confirm the reversibility of inhibition by cysmethynil, preformed EI* was diluted into

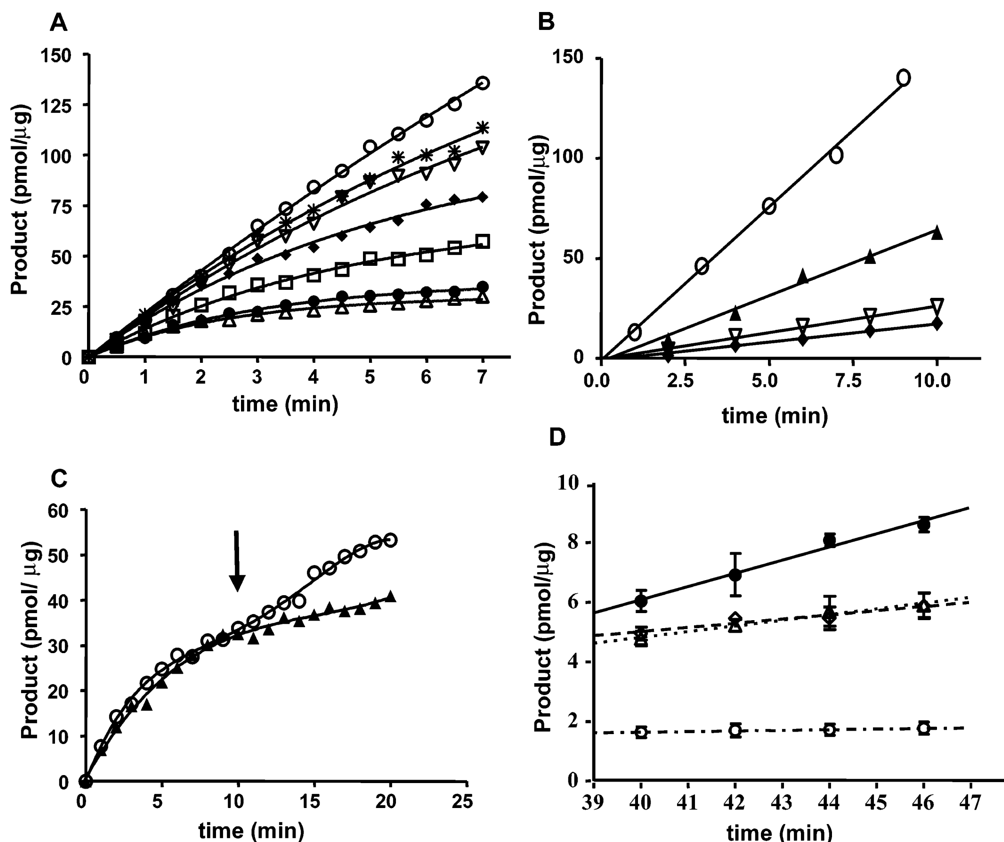

FIGURE 2: Analysis of time-dependent inhibition of Icmt by cysmethynil. (A) Progress curves of methylation of FKRasCOO$^-$ illustrating time-dependent inhibition of Icmt in presence of cysmethynil. The Icmt assay (see Experimental Procedures) was carried out in the presence of 2 μM FK-RasCOO$^-$, 5 μM AdoMet, and the specified concentration of cysmethynil; at the specified times, aliquots were withdrawn, reactions were terminated, and the product was identified. Data represent the means of duplicate determinations from a single experiment that is representative of three such experiments. Data were fitted to eq 3. (B) Progress curves of methylation of FK-RasCOO$^-$ in presence of "classical" Icmt inhibitors. The Icmt reaction were performed as described for panel A in the presence of vehicle (DMSO) (○), 15 μM FTA (▲), 100 μM AFC (▽), or 40 μM AdoHCy (◆). Data represent the means of four determinations from two separate experiments and were fitted to a line. (C) Distinguishing between reversible and irreversible inhibition of Icmt by cysmethynil. The Icmt reaction was performed as described for panel A in the presence of 2 μM cysmethynil. Ten minutes after reactions were initiated, reaction mixtures were supplemented with either vehicle [DMSO (○)] or with additional FK-RasCOO$^-$ substrate to a final concentration of 8 μM (▲). Data represent the means of four determinations from two separate experiments. Data were fitted to eq 2 to extract kinetic parameters. (D) Icmt velocity curves from 40 to 46 min after dilution of EI*. Concentrations of cysmethynil are indicated and represent pre- and postdilution concentrations as described in Experimental Procedures [0-0 (●), 1 and 1 μM (△), 50 and 1 μM (◇), and 50 and 50 μM (○)]. The values represent the means ± the standard error of the mean determined from duplicate determinations of two independent experiments.

buffer lacking inhibitor and enzyme activity monitored. As seen in Figure 2D, EI* diluted into buffer had the same activity as Icmt assayed in the presence of a diluted concentration of cysmethynil. The recovery of activity compared to the inhibited control in the presence of persistent concentrations of the inhibitor confirms the reversibility of cysmethynil. This result was expected as there are no highly reactive moieties on this compound.

Having confirmed time-dependent inhibition of Icmt by cysmethynil, we then determined the rate constants associated with this property. In the most common mechanism through which time-dependent inhibition is manifest (so-called mechanism B), (*21*), the initial velocity is a function of the inhibitor concentration and the apparent first-order rate constant varies as a hyperbolic function of inhibitor concentration. Thus, there is an initial rapid equilibrium between the enzyme and the inhibitor in which the EI complex is rapidly formed, after which the complex undergoes a slow isomerization reaction diagrammed as follows:

$$E + I \leftrightarrows EI \leftrightarrows EI^* \qquad (I)$$

The forward and reverse rate constants for EI formation are k_1 and k_2, respectively, and for the second equilibrium are k_3 and k_4, respectively. The overall inhibition constant (K_i^*) is thus

$$K_i^* = [E][I]/([EI] + [EI^*]) = K_i k_4/(k_3 + k_4) \qquad (6)$$

where $K_i = k_2/k_1$. In this mechanism, the EI complex is formed rapidly, but this complex subsequently isomerizes slowly to a tighter EI* complex (*21*, *26*). By fitting the data to eq 3, we analyzed the data shown in Figure 2A to give values for v_o, v_s, and k_{obs} for each inhibitor concentration (see Experimental Procedures). Via analysis of k_{obs} as a function of inhibitor concentration, it became clear that values of k_{obs} reached a plateau at high cysmethynil concentrations (Figure 3). These results indicated that the type of inhibition is indeed that of a rapid initial association followed by formation (presumably via some sort of conformational change in the enzyme) of the high-affinity EI* complex. When the values for v_o and the corresponding inhibitor concentrations from these experiments are fitted to eq 1, the

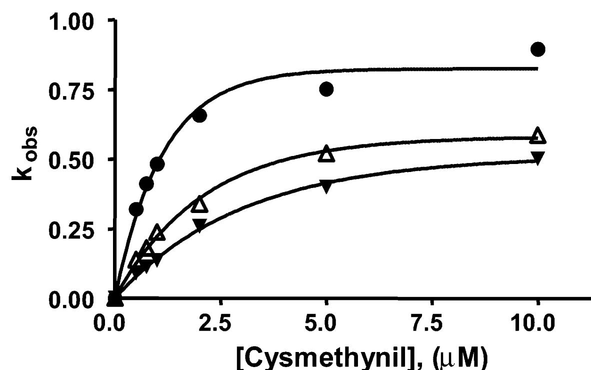

FIGURE 3: Analysis of k_{obs} as a function of cysmethynil concentration at three farnesylated substrate (FK-RasCOO$^-$) concentrations: 1 (●), 2 (△), and 4 μM (▼). k_{obs} was calculated by nonlinear regression of progress curves from Figure 2A as described in Experimental Procedures. The data were fitted to eq 4. The data shown represent the mean of duplicate determinations from a single experiment, which is representative of three such experiments.

Table 1: Experimental Kinetic Constants for Time-Dependent Inhibition of Icmt by Cysmethynil[a]

constant	experimental value
K_i	2.39 ± 0.02 μM
K_i^*	0.113 ± 0.02 μM
k_4	0.053 ± 0.003 min^{-1}
k_3	0.86 ± 0.06 min^{-1}

[a] The values were determined from progress curves as described in the legend of Figure 2A. The values represent the mean ± the standard error of the mean determined for seven progress curves.

K_i for the first, rapid binding, phase was calculated to be 2.39 ± 0.02 μM, which is in close agreement with the experimentally determined K_i from the steady-state measurement described above (and see Figure 1). The value for K_i^* (i.e., the high-affinity form) was then calculated by replacing v_o with v_s and K_i with K_i^* in eq 1 and determined to be 0.113 ± 0.013 μM. When the data were fit to eq 4, the rate of conversion (k_4) of the EI* complex to EI was also determined from these data (Figure 3); this value was 0.053 ± 0.003 min^{-1}, corresponding to a half-life for the existence of the high-affinity complex of ~15 min. The rate of conversion of the EI complex to the EI* complex, k_3, calculated by using eq 5 and the value of k_3, was determined to be 0.86 ± 0.06 min^{-1}, with the $t_{1/2}$ for this transition thus being 0.8 min. A summary of the kinetic constants related to inhibition of Icmt by cysmethynil is presented in Table 1. As an independent verification of the K_i^* value calculated from the reaction progress data, Icmt was preincubated with increasing concentrations of cysmethynil for 15 min at 37 °C to allow the formation of the EI* complex, after which the rate of product formation (v_s) was determined in assay solutions containing the two substrates and the same inhibitor concentrations. Calculation of K_i^* using these data and eq 1 yielded a K_i^* value of 0.138 ± 0.012 μM (data not shown), which is in close agreement with the K_i^* value determined from the time-dependent analysis described above.

Substituents on the Indole Nitrogen of This Class of Icmt Inhibitors Are Critical for Manifestation of Time-Dependent Inhibition. Cysmethynil was identified in a screen of a diversity library that contained a larger group of compounds assembled on an indole core. Of these compounds, cysmethynil was the most potent and hence was chosen for further study (*18*). We have now examined a select set of these compounds to determine whether they exhibited time-dependent inhibition and discovered that the substituent on the indole nitrogen plays a significant role in this behavior. Compounds containing a variety of R1 substitutions on the indole nitrogen (Table 2) were assayed for inhibitory potential either under standard conditions or following preincubation with Icmt for 30 min at 37 °C (see Experimental Procedures). When R1 was a small carbon chain such as isobutyl or cyclopropyl, the compounds exhibited essentially the same IC_{50} value with or without preincubation of the compound with the enzyme (Table 2). However, when the R1 substituent was a longer carbon chain or a more hydrophobic moiety such as hexyl, octyl (to yield cysmethynil), benzyl, 3-trifluoromethyl benzyl, or naphthyl, time-dependent inhibition was observed (Table 2). Thus, a decrease in the length versus hydrophobicity of the indole nitrogen substituent is accompanied by loss of the time-dependent properties of this indole class of Icmt inhibitors.

DISCUSSION

It is now well-established that Icmt-catalyzed methylation of prenylated proteins is important for the correct localization and function of a number of CaaX-containing proteins (*8*, *27*). However, the contribution of carboxylmethylation to specific processes has in many cases been difficult to experimentally address given the unavailability of specific inhibitors of this process. The only inhibitors available prior to the discovery of cysmethynil were either structural mimics

Table 2: Indole Nitrogen Substituents Influence the Manifestation of Time-Dependent Inhibition of Icmt[a]

R_1	IC_{50}			*		F F F		
	15.7± 2μM	9.1± 1.8μM	7.4± 0.4μM	2.1± 0.9μM	6.4± 2μM	8± 4.5μM	17.2± 4.3μM	No PI
	17.7± 4.7μM	9.5± 1.2μM	1.7± 0.08μM	0.29± 0.03μM	2.4± 0.7μM	0.71± 0.09μM	1.43± 0.2μM	PI

[a] The IC_{50} values for inhibition of Icmt by the indicated indole compounds, including cysmethynil (asterisks) were determined with (PI) or without (no PI) preincubation of the enzyme with the compound prior to the initiation of the assay. Data represent duplicate determinations from three experiments. See Experimental Procedures for details.

of the prenylcysteine substrate or the product AdoHcy (*24*, *28*−*30*). A number of studies have attempted to inhibit Icmt activity in cells using these prenylcysteine analogues or pharmacological compounds that elevate the level of AdoHcy (*19*, *27*, *30*, *31*), but the poor specificity of these approaches has made it difficult to attribute specific results to inhibition of Icmt (*8*). Knockout of the *Icmt* gene in mice has demonstrated the importance of this enzyme in embryonic development (*32*, *33*) and in oncogenesis (*17*), but this approach is viable for only the limited number of cell types derived from the knockout mice. Hence, the identification of cysmethynil as a potent, selective, cell-active inhibitor of Icmt has provided a unique tool with which to interrogate biologies associated with C-terminal methylation of prenylated proteins, and it therefore becomes important to understand the inhibitory properties of this compound.

Because of our finding that the IC_{50} of cysmethynil decreased when Icmt was preincubated with the compound (*18*), we investigated the properties of time-dependent inhibition of Icmt by the compound. The experiments described herein demonstrate that time-dependent inhibition did indeed occur and could be represented by a two-step process. This assessment is based on the dependence of the initial rates on the inhibitor concentration and was further characterized by the hyperbolic dependence of k_{obs} on the concentration of the inhibitor. In this two-step process, the first step is represented by formation of an EI complex with a K_i value for cysmethynil of 2.39 ± 0.02 μM. This complex then undergoes an isomerization to form a tighter EI* complex with an overall dissociation constant for cysmethynil of 0.11 ± 0.01 μM. An important feature of time-dependent inhibitors is their time of dissociation from the enzyme. In the case described here with cysmethynil and Icmt, the half-life for the conversion of the EI* complex back to EI is ∼15 min. In biological settings and in pharmacological applications, this is considered an advantage because the enzyme is inhibited for a relatively long period.

Another important finding in this study is that substituents on the indole nitrogen of the compound play a crucial role in the manifestation of time-dependent inhibition by this class of compounds. When this substituent is small and/or weakly hydrophobic, no time dependence of inhibitory property is observed. As the hydrophobicity of the indole nitrogen substituent is increased, the potency of inhibition increases and time-dependent behavior is observed. These results argue in favor of this position playing an important role in the mechanism of inhibition and formation of the EI* complex and provide a framework for potential identification of even more efficacious inhibitors of Icmt.

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