COMMUNICATIONS

Inhibition of Membrane-Associated Methyltransferases by a Cholesterol-Based Metal Chelator

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We have designed, synthesized, and characterized a metal chelating compound that is based on the structure of cholesterol and contains the high affinity metal chelating group, lysine nitrilotriacetic acid (Lys-NTA). Using the enzyme isoprenylcysteine carboxylmethyltransferase (Icmt) from yeast as a model integral membrane metalloenzyme, we find that this agent potently inhibits Icmt activity with an IC_{50} value between 35 and 75 μM, which is at least 40 times more potent than the best known Icmt metal chelating inhibitor, Zincon. We propose that the rigid hydrophobic cholesterol moiety promotes partitioning into the membrane, enabling the metal-binding NTA group(s) to inactivate the enzyme by metal chelation. Because this compound is based on a naturally occurring membrane lipid and appears to chelate metals buried deeply within water insoluble environments, this agent may also be useful as a general tool for identifying previously unappreciated metal dependencies of other classes of membrane proteins.

Metalloproteins are essential to biological systems, playing critical roles in processes ranging from oxygen transport to signal transduction. The metals within these proteins can serve as structural components or as active site catalytic centers. Although many identified metalloproteins are membrane-spanning proteins, possessing one or more hydrophobic domains, the relative water solubility of standard metal chelators used to probe membrane protein function suggests that many other membrane proteins may have gone unrecognized as metalloproteins. In this study, we report the development of a cholesterol-based chelator that may serve as an excellent tool for identifying the metal dependence of membrane proteins. This molecule (**7**) contains the high affinity metal chelating group, lysine nitrilotriacetic acid (Lys-NTA), covalently linked via an N^{ϵ}-carbamoyl linkage to cholesterol through the 3-hydroxyl on ring A (Chart 1). Other classes of chelator lipids coupled to Lys-NTA, such as NTA-dioctadecylamine (NTA-DODA) (*1*) or imidodiacetic acid (IDA) (*2*), have been synthesized and used previously for targeting biomolecules to artificial membranes and for organizing both soluble and membrane-associated histidine-tagged biomolecules at lipid interfaces to form two-dimensional arrays (*1*−*15*).

We used the isoprenylcysteine carboxylmethyltransferase (Icmt) from *S. cerevisiae*, Ste14p, as the model enzyme for this study. Ste14p is a 26 kDa endoplasmic reticulum (ER) membrane-spanning enzyme responsible for the posttranslational carboxylmethylation of CaaX motif proteins (*16*−*19*). Preliminary evidence suggests that Ste14p and its mammalian Icmt counterparts are dependent upon metals for activity (*20*, *21*). Although concentrations of EDTA up to 100 mM have no effect on activity, treatment of crude membranes with relatively high concentrations (10−50 mM) of *o*-phenanthroline eliminate the catalytic activity, presumably by chelation and perhaps removal of the metal from the enzyme (*20*, *21*). Lower concentrations of the more hydrophobic chelator compound 2-[5-(2-hydroxy-5-sulfophenyl)-3-phenyl-1-formazyl]benzoate (Zincon) were more effective, demonstrating 50% inhibition at approximately 3 mM (*21*). Taken together, these data suggest that more membrane-permeable, polycyclic, hydrophobic chelators would be increasingly effective at inhibiting these methyltransferases. In particular, we hypothesized that chelating moieties linked to naturally occurring membrane components may be most efficacious.

The compounds shown in Chart 1 were synthesized (*22*) to determine the molecular properties that are required to successfully inhibit Ste14p activity (see Supporting Information for the detailed synthesis of cholesterol-Lys-NTA (**7**)). Methyltransferase assays were performed essentially as described (*23*−*26*) with minor modifications. The assay mixture contained a total volume of 60 μL and a final Tris-HCl concentration of 100 mM, pH 7.5. All reactions contained 20 μM *S*-adenosyl-[^{14}C-*methyl*]-methionine (SAM) and 5 μg of membrane protein from Ste14p-overexpressing yeast strains (*21*). Inhibition curves were generated by varying the amount of potential inhibitor compound in each reaction

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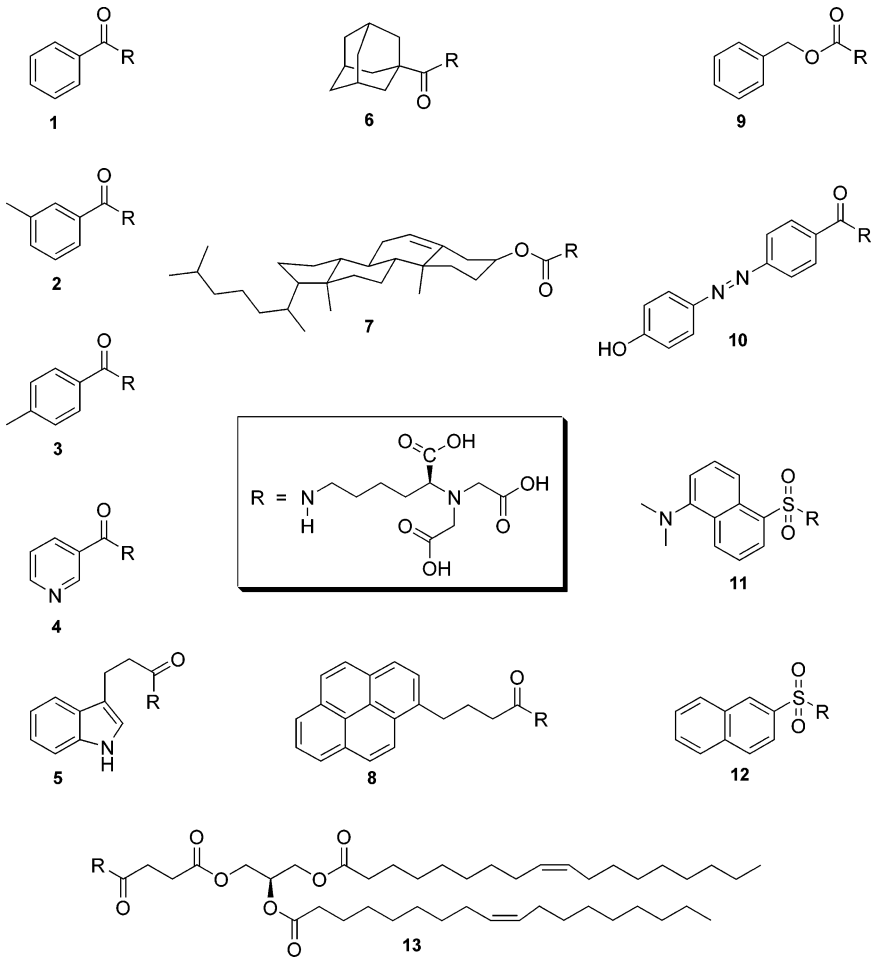
Chart 1. Metal-Chelating Organic Compounds Tested as Inhibitors on Ste14p Activity in Vitro[a]



[a] Benzoic-Lys-NTA (**1**); *m*-toluic-Lys-NTA (**2**); *p*-toluic-Lys-NTA (**3**); nicotinic-Lys-NTA (**4**); indole-3-acetic-Lys-NTA (**5**); adamantane-Lys-NTA (**6**); cholesterol-Lys-NTA (**7**); 1-pyrenebutyric-Lys-NTA (**8**); benzylcarbamoyl-Lys-NTA (**9**); azo-Lys-NTA (**10**); dansyl-Lys-NTA (**11**); 2-naphthalenesulfonyl-Lys-NTA (**12**); 1,2-dioleoyl-sn-glycero-3-[*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (DOGS)-NTA (**13**) (Avanti Polar Lipids, Inc.)

in the presence of *N*-acetyl-*S*-farnesyl-L-cysteine (AFC), the methyl accepting substrate, at 33 μM, which is twice the K_m for the yeast Ste14p enzyme in membranes. Membranes were preincubated for ~5 min with inhibitor compounds on ice prior to the addition of AFC and SAM. The reactions were then incubated at 30 °C for 30 min and stopped with the addition of 50 μL of 1 M NaOH/1% (v:v) SDS. One hundred microliters of this mixture was spotted on folded filter paper (5.5 cm × 1.5 cm) and lodged in the neck of a scintillation vial containing 10 mL of scintillation fluid. Hydroxide ion forms a tetrahedral intermediate with the newly formed ^{14}C-methyl ester on the methyl acceptor. This intermediate then eliminates ^{14}C-methanol that diffuses into the scintillation fluid below. The filters were pulled out after 2−3 h, and the vials were shaken well and counted in a liquid scintillation analyzer.

In this study, we found that only cholesterol-Lys-NTA (**7**) was effective in inhibiting the in vitro methyltransferase activity of Ste14p (Table 1, Figure 1). The Ste14p protein used in these experiments contains a 10 histidine repeat at the N-terminus followed by a triply iterated *myc* epitope tag (His-Ste14p) (*21*). These tags were engineered to aid in purification and identification of Ste14p by immunoblot analysis. The inhibition by each compound is shown in Table 1 and is represented as the percent methyltransferase activity remaining upon treatment with 1 mM of the respective compound compared

Table 1. Percent Activity of Ste14p in Vitro in the Presence and Absence of 1 mM Inhibitor Compounds

compound no.	percent activity	compound no.	percent activity
no inhibitor	100 ± 2.6	**7**	5.8 ± 2.5
1	108 ± 2.2	**8**	108 ± 1.2
2	111 ± 1.9	**9**	112 ± 1.3
3	108 ± 1.7	**10**	112 ± 4.1
4	111 ± 2.2	**11**	104 ± 1.9
5	108 ± 3.4	**12**	122 ± 4.6
6	107 ± 1.8	**13**	76.2 ± 4.6

to the untreated control reaction. We further examined the concentration dependence of inhibition by **7** on methyltransferase activity and determined the IC_{50} value to be ~75 μM (Figure 1), which is nearly 40 times more potent than the best known Icmt inhibitor, Zincon. The relative potency of this compound compared to EDTA and *o*-phenanthroline can be attributed to both the hydrophobicity of the cholesterol moiety ligated to the chelating agent Lys-NTA and the rigidity of the fused ring system. We propose that the rigid hydrophobic cholesterol moiety promotes partitioning into the membrane, enabling the metal-binding NTA groups to inactivate the enzyme by metal chelation. The only other compound that demonstrated any inhibitory ability (~25% inhibition; Table 1) was the commercially available DOGS−NTA (**13**), another compound that resembles a naturally occurring membrane lipid. The relative ineffectiveness of DOGS−NTA as an inhibitor may be due to the flexibility of this

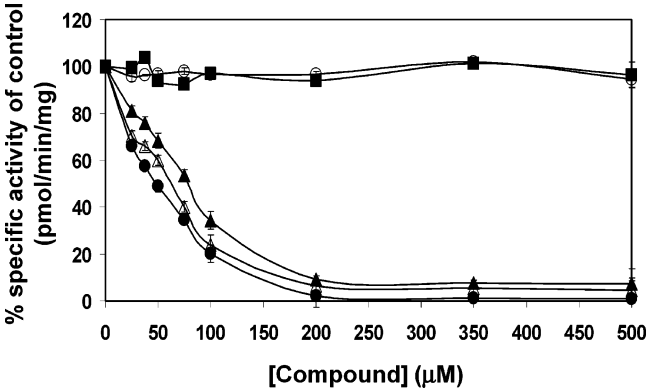


Figure 1. Concentration dependent inhibition of Ste14p-dependent methyltransferase activity by compound **7** and related compounds. Three Ste14p variants were used: His-Ste14p, untagged Ste14p and Ste14p^{C99S,C121S,C126S}. Assays were performed with 5 μg crude membrane protein expressing one of these variants in the presence of 33 μM *N*-acetyl-S-farnesyl-L-cysteine (AFC) and the activity was quantified by the in vitro vapor diffusion methyltransferase assay (*23, 26, 28*). To normalize for batch to batch and variant to variant differences in enzymatic activity, the inhibition data is presented as the percent of the control reactions that lacked inhibitor. His-Ste14p in the presence of **7** (▲); Cholesterol-Lys-NTA trimethyl ester (○); or cholesterol (■). Compound **7** incubated with untagged Ste14p (●) or Ste14p^{C99S,C121S,C126S} (△). Each experiment was performed in duplicate a minimum of two times. Error bars report standard error.

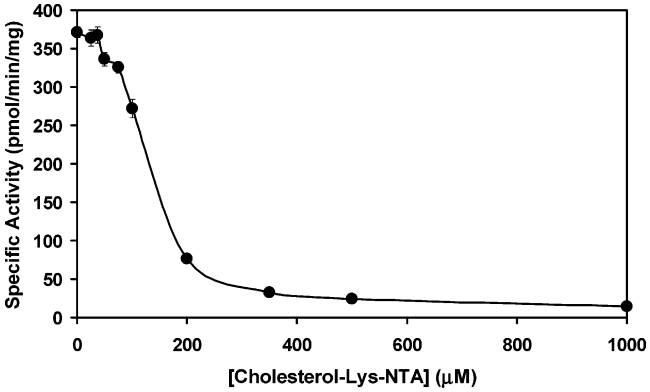


Figure 2. Concentration dependent inhibition of His-hIcmt-dependent methyltransferase activity by compound **7**. Assays were performed with 5 μg of crude membrane protein expressing His-hIcmt in the presence of 33 μM AFC, and the activity was quantified by the in vitro vapor diffusion methyltransferase assay. Each experiment was performed in duplicate a minimum of two times. Error bars are reported as standard error.

compound that may preclude access of the NTA group to the hydrophobic metal binding region of the protein.

Because the Ste14p protein used in these experiments contains a 10 histidine repeat at the N-terminus followed by a triply iterated *myc* epitope tag, we needed to ensure that the inhibition we observed with the cholesterol-Lys-NTA compound (**7**) was not due to interaction with the tags on Ste14p. Therefore, we constructed and expressed an untagged version of the Ste14p protein and performed similar methyltransferase assays in the presence and absence of **7**. These results demonstrated that the untagged wild-type protein is also inhibited by **7** in a concentration dependent manner with an IC_{50} of ~45 μM (Figure 1), suggesting that interaction of **7** with the tags is not responsible for the inhibition.

To further substantiate our hypothesis that **7** is inhibiting via metal chelation, we examined the effects of unmodified cholesterol and cholesterol-Lys-NTA trimethyl ester as inhibitors. In the latter compound, the carboxylic acids in the NTA portion of the molecule are methyl-esterified and thus unable to engage in metal chelation. The addition of increasing concentrations of either cholesterol or cholesterol-Lys-NTA trimethyl ester to the reaction had no inhibitory effect on Ste14p methyltransferase activity (Figure 1). These data further support the hypothesis that the inhibition mechanism of **7** is via metal chelation. An alternative hypothesis suggesting that Ste14p methyltransferase inhibition arises from a detergent effect of the hydrophobic NTA derivatives is not supported by our findings since the DOGS−NTA compound, a superior detergent to cholesterol-Lys-NTA, only inhibits ~25% of the methyltransferase enzymatic activity at 1 mM (Table 1).

Cysteine and histidine residues are common chelating ligands in proteins. To assess the importance of cysteines in the activity of Ste14p as well as in the ability of **7** to inhibit activity, a mutant of Ste14p was constructed that had all three naturally occurring cysteines mutagenized to serine (Ste14p^{C99S,} Ste14p^{C121S,} Ste14p^{C126S}). This cysteine-less Ste14p variant was expressed equally to the untagged wild-type protein as determined by immunoblot analysis (see Supporting Information). Our data further demonstrate that **7** inhibits the cysteine-less mutant with an IC_{50} of ~35 μM, a value comparable to that for the untagged wild-type enzyme (Figure 1). Taken together, these data suggest that the cysteine residues in Ste14p are not essential for activity and that other amino acids in Ste14p must be ligated to the metal ion in the protein structure.

We further examined the ability of **7** to inhibit the activity of the human homologue of Ste14p, hIcmt. Ste14p and hIcmt share 41% identity and 63% similarity overall, suggesting that their three-dimensional structures and mechanisms of action are similar. In fact, human Icmt expressed in yeast functionally complements a Δ*ste14* deletion strain (*27*). By analogy to the His-Ste14p experiments, an epitope tagged variant of hIcmt (His-hIcmt) containing a 10 histidine repeat at the N-terminus followed by a triply iterated *myc* epitope tag was expressed in *S. cerevisiae* and assayed for activity in the presence and absence of increasing concentrations of **7** (Figure 2) (*21*). The calculated IC_{50} for inhibition of His-hIcmt with **7** is ~135 μM (Figure 2). These data not only suggest that the human and yeast enzymes both have similarly accessible metal ions and, thus, three-dimensional structures, they also show that **7** should prove to be a excellent tool for studying the metal dependence of other members of the Icmt family of enzymes.

We report here that the novel compound cholesterol-Lys-NTA (**7**) exhibits great potential as a tool for characterizing the metal dependence of the Icmt family of enzymes. Although outside the scope of this initial study, complete certainty of the efficacy of **7** as a membrane metalloenzyme probe will come with the successful identification of the chelated metal. We are currently developing a strategy to extract the cholesterol−Lys-NTA−metal complex from reconstituted vesicles containing purified Ste14p (*21*) and are seeking to identify the metal by inductively coupled plasma mass spectrometry. Because **7** is based on a naturally occurring membrane lipid and appears to chelate metals buried deeply within water insoluble environments, this agent may also be useful as a general tool for identifying previously unappreciated metal dependencies of other classes of membrane proteins.

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Supporting Information Available: The chemical synthesis of the compound **7** as well as a description of the cloning procedures, immunoblot assays, and enzymatic assay is available free of charge via the Internet at http://pubs.acs.org.

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