Design of Allele-Specific Protein Methyltransferase Inhibitors

ARTICLE *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · DECEMBER 2001

Impact Factor: 12.11 · DOI: 10.1021/ja011423j · Source: PubMed

Design of Allele-Specific Protein Methyltransferase Inhibitors

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Received June 11, 2001

Abstract: Protein arginine methyltransferases, which catalyze the transfer of methyl groups from *S*-adenosylmethionine (SAM) to arginine side chains in target proteins, regulate transcription, RNA processing, and receptor-mediated signaling. To specifically address the functional role of the individual members of this family, we took a "bump-and-hole" approach and designed a series of N^6-substituted *S*-adenosylhomocysteine (SAH) analogues that are targeted toward a yeast protein methyltransferase *RMT1*. A point mutation was identified (E117G) in Rmt1 that renders the enzyme susceptible to selective inhibition by the SAH analogues. A mass spectrometry based enzymatic assay revealed that two compounds, N^6-benzyl- and N^6-naphthylmethyl-SAH, can inhibit the mutant enzyme over the wild-type with the selectivity greater than 20. When the E117G mutation was introduced into the *Saccharomyces cerevisiae* chromosome, the methylation of Npl3p, a known in vivo Rmt1 substrate, could be moderately reduced by N^6-naphthylmethyl-SAH in the resulting allele. In addition, an N^6-benzyl-SAM analogue was found to serve as an orthogonal SAM cofactor. This analogue is preferentially utilized by the mutant methyltransferase relative to the wild-type enzyme with a selectivity greater than 67. This specific enzyme/inhibitor and enzyme/substrate design should be applicable to other members of this protein family and facilitate the characterization of protein methyltransferase function in vivo when combined with RNA expression analysis.

Introduction

Despite the large number of bioactive compounds that have been identified from screening synthetic and natural product collections, it remains challenging to identify potent and selective inhibitors of a given protein of interest. The problem of improving inhibitor selectivity is especially acute in large protein families with highly conserved active sites. One solution to this problem is through a "bump-and-hole" approach in which a mutation is introduced to the protein active site that renders the enzyme (E*) susceptible to selective inhibition by a small molecule (L*) (Figure 1).[1,2] This approach has been successfully implemented to kinases,[3] kinesin and myosin motors,[4] nuclear hormone receptors,[5] and human growth hormone receptors.[6] The major advantage of this approach over a traditional genetic strategy is that greater control over the time and extent of protein inhibition can be achieved.

S-Adenosylmethionine (SAM) dependent protein arginine methyltransferases (PRMTs) are involved in transcriptional regulation, RNA processing, and receptor-mediated signaling in eukaryotic organisms.[7] Several arginine methyltransferases have recently received considerable attention including the yeast Rmt1/Hmt1[8] and Hsl7,[9] the rat PRMT1[10] and PRMT3,[11] and the human CARM1[12] and JBP1,[13] yet their in vivo functions are still far from clear. The predominant protein arginine methyltransferase in *S. cerevisiae*, *RMT1/HMT1* represents an ideal entry point into the study of this important gene family. For example, *RMT1* is responsible for the synthesis of 85% of

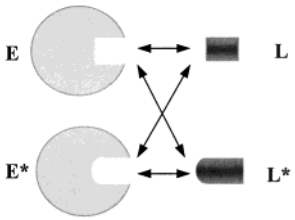

Figure 1. A schematic representation of a "bump-and-hole" approach. E, E*, L, and L* denote wild-type enzyme, engineered enzyme, natural ligand (inhibitor or substrate), and "bumped" ligand, respectively.

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10.1021/ja011423j CCC: $20.00
Published on Web 11/01/2001

A

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Yeast Rmt1     109 ITLLRGKLEDVHLP 122
Rat PRMT3      303 IVLIKGKIEEVSLP 316
Rat PRMT1      121 VTIIKGKVEEVELP 134
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B

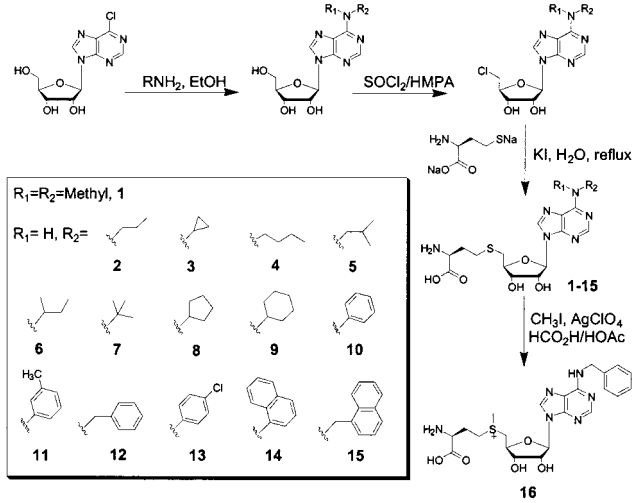
Figure 2. (A) The sequence alignment of three methyltransferase fragments where the conserved Glu that make contacts with SAH were underlined. (B) The crystal structure of the active site of the Prm3-SAH complex. E311 forms a hydrogen bonding with N^6 of SAH.

N^G,N^G-dimethylarginine found inside the cell. It methylates an mRNA binding protein Npl3p both in vitro and in vivo, and the methylation is important for the export of Npl3p from nucleus.[14] The discovery of cell permeable selective substrates/inhibitors of Rmt1 should provide a useful tool for in vivo studies of this enzyme, including identification of its downstream substrates. Here, we present an approach to the discovery of allele-specific inhibitors of protein arginine methyltransferases and preliminary functional studies of these inhibitors in *Saccharomyces cerevisiae* in vivo.

Results

Selective Substrate/Inhibitor Design and Synthesis. All PRMTs utilize *S*-adenosylmethionine (SAM) as a cofactor in the methyl-transfer reaction, and share very high homology in the SAM binding domains (Figure 2A).[15] The product of the methyltransferase reaction, *S*-adenosylhomocysteine (SAH), functions as a nonselective inhibitor. Structural studies have indicated that there are four conserved motifs (designated as I, post I, II, and III) in the SAM-binding domains of all protein arginine methyltransferases. The structural module for SAM binding is formed mainly by motifs I and post I. A crystal structure[16] of a rat methyltransferase PRMT3 catalytic core-SAH complex has been solved recently (Figure 2B), providing the first glimpse into the cofactor-binding geometry and the possible underlying methyl-transfer mechanism. The residues that interact with SAH are located largely in the three N-terminal helices of the protein. Furthermore, nitrogens N^6, N^1, N^7 of the adenosine ring interact with the side chain of E311 in the loop

Scheme 1



of the post I region, the main chain NH of I310, and a water molecule, respectively. It is expected that specific enzyme/substrate or enzyme/inhibitor pairs can be designed by modifying the adenosine-E311 interactions in the adenosine-binding pocket (Figure 2B). Since this site is remote from the reactive sulfonium group of the SAM molecule, it should not adversely affect the methyl-transfer ability of the resulting engineered enzyme. Thus, a "bump-and-hole" strategy consisting of two steps was employed in the design of Rmt1 selective inhibitors/substrates. First, a single amino acid point mutation (E117G, corresponding to E311G in Prmt3, Figure 2A)[17] was made to Rmt1 to create a "hole" around the N^6-position of the adenosine ring (E* in Figure 1). Second, a bulky substitutant was placed on the N^6-position of the adenosine ring to create a "bump" on the SAM or SAH ligands (L* in Figure 1). It was anticipated that this mutant enzyme, but not the wild-type transferase or its homologues, would accept a "bumped" SAM analogue such as N^6-benzyl-*S*-adenosylmethionine (**16**) (Scheme 1) as a cofactor. As a result, only the substrates of the mutant enzyme should be methylated in the presence of an isotopically labeled N^6-benzyl SAM analogue. In addition, the mutant enzyme may be selectively inhibited by the "bumped" SAH analogues in the presence of the wild-type or its homologous enzymes.

A series of N^6-substituted SAH analogues (**1**−**15**) were synthesized[18] in three steps starting from 6-chloroadenosine (Scheme 1). A variety of groups were introduced into the N^6-position of the adenosine ring through the nucleophilic substitution of chloro by alkyl-, cycloalkyl-, benzyl-, and arylamines. Screening the SAH analogues with the substituents of different size, shape, and rigidity should yield a complementary ligand (L*) for the engineered hole in the active site (E*). Due to the resulting sulfonium chiral center,[19] a diastereomeric mixture of N^6-benzyl-*S*-adenosylmethionine (**16**) was synthesized by alkylating the sulfur atom of N^6-benzyl-*S*-adenosyl-homocysteine (**12**) with excess methyl iodide in the presence of $AgClO_4$. It should be noted that only one N^6-benzyl-SAM diastereomer is active as the methyl donor in the methyltransferase catalyzed reactions.[20]

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Table 1. Methyltransferase Activity of Wild-Type and Mutant Enzyme

cofactor	methyl-transferase	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}min^{-1}$)
SAM	WT	1.1	6.7	6.1×10^6
	E117G	3.2	4.9	1.5×10^6
Bzl-SAM (**16**)	WT	>100	<0.02	<200
	E117G	18.0	0.23	1.3×10^4

Kinetic Studies and Inhibitor Screening. The catalytic activities of the Glu[117]→Gly mutant and wild-type enzymes were investigated by using a mass spectrometry based assay with a synthetic peptide (H-GGFGGRGGFG-NH_2) as the substrate.[21] The relative rates of methylation for wild-type or mutant enzyme catalyzed reactions were followed with either SAM or Bzl-SAM (**16**) as the cofactor. The corresponding k_{cat} and K_m values for each individual enzyme/substrate pair were derived from curve-fitting the plots with the Michaelis−Menten equation and listed in Table 1. The k_{cat}/K_m of the E117G mutant was determined to be 1.5×10^6 M^{-1} min^{-1}, about 4-fold less active than the wild-type enzyme. However, when the "bumped" SAM analogue, **16**, was used as the cofactor, the methylation reaction catalyzed by the wild-type enzyme could not be observed.[22] In contrast, the E117G mutant enzyme catalyzed the methyltransfer reaction in the presence of analogue **16** with a k_{cat}/K_m only 115-fold lower than that for SAM. This differential catalytic activity is similar to that found in other orthogonal enzyme−substrate pairs. For example, in the work on engineering of the orthogonal kinase/ATP pair the k_{cat}/K_m of the engineered Src (V323A, I338A)/N^6-cyclopentyl-ATP pair is about 50-fold lower than that of the natural Src/ATP pair.[23] Thus, the "bumped" cofactor (**16**) is a viable substrate for the mutant enzyme.

To determine whether the E117G mutant enzyme could be selectively inhibited by "bumped" SAH analogues, an in vitro inhibition assay was carried out for compounds **1**−**15** in the presence of either wild-type or mutant enzyme. Two moderately selective inhibitors, benzyl-SAH (**12**) and naphthylmethyl-SAH (**15**), were identified (Table 2). This suggests that the substituents with a rigid aromatic ring and a flexible methylene linker may provide the optimum fit for the engineered "hole" in the active site. Both compounds preferentially inhibit the mutant enzyme with K_i values of 4.4 and 5.0 μM, respectively, and selectivity factors greater than 20 relative to the wild-type enzyme.[24] For comparison, two known methyltransferase inhibitors, SAH and sinefungin, preferentially inhibit the wild-type enzyme over the mutant enzyme with selectivity of 0.22 and 0.16, respectively (Table 2). A radioactive inhibition assay was also carried out for **15** with [^{14}C]-SAM as the methyl donor and an in vivo Rmt1 substrate Npl3p in yeast cell extracts as the methyl acceptor. After separation by gel electrophoresis, the extent of Npl3p methylation was quantified by the incorporation of ^{14}C in its corresponding protein bands on the gel. Again, analogue **15** was able to preferentially inhibit the E117G mutant catalyzed reaction over the wild-type with an IC_{50} of 100 μM (Figure 3).

Table 2. Inhibition of the Wild-Type and Mutant Rmt1 Enzymes by SAH Analogues

analogue	K_i (μM)[a] WT	K_i (μM)[a] E117G	selectivity[b]
SAH	1.6	7.2	0.22
sinefungin	0.6	3.7	0.16
1	>100	350	>0.29
2	>100	80	>1.2
3	>100	100	>1.0
4	>100	53	>1.9
5	>100	110	>0.91
6	>100	350	>0.29
7	>100	530	>0.19
8	>100	510	>0.20
9	>100	240	>0.42
10	>100	50	>2.0
11	>100	100	>1.0
12	>100	4.4	>23
13	>100	80	>1.3
14	>100	>100	ND
15	>100	5.0	>20

[a] K_i values were calculated from $K_i = IC_{50}/(1 + ([S]/K_m))$; [b] Selectivity was defined as K_i(wt)/ K_i(mt); ND, not determined.

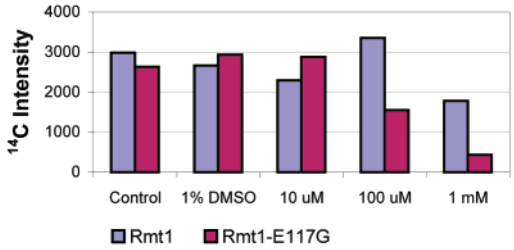

Figure 3. Inhibition of Npl3p methylation by **15** in the cell extracts.

Cellular Effects of the Selective Inhibitors. To demonstrate that the N^6-substituted SAH analogues can exert their inhibitory effect on the methylation process catalyzed by Rmt1 in vivo, we constructed a yeast strain *rmt1-as1* in which the wild-type *RMT1* gene on the chromosome was replaced by an *RMT1-E117G* mutant gene through a homology recombination technique.[25] Briefly, the *RMT1-E117G* gene was placed on a URA3-containing integration vector pRS306. The vector was linearized and integration was achieved through homologous recombination and a uracil selection. The removal of the vector from the chromosome was achieved through self-arrangement and selection with 5-FOA. The strains that carry the *RMT1-E117G* alleles were identified through sequencing of the PCR products of the genomic DNA.

Treatment of both yeast strains with 10 μM to 1 mM inhibitor **15** had no apparent effect on yeast growth, although the mutant strain grows a bit slower than the wild-type (Figure 4). This is consistent with the previous observation that Rmt1 is a nonessential gene. The *RMT1* null mutant has no apparent defects in either mRNA nuclear export or nuclear protein localization.[14] However, when cells treated with 1 mM **15** were lysed and the lysates were probed with an antibody that selectively recognizes the methylated form of an Rmt1 substrate, Npl3p, the methylation of Npl3p in the mutant allele was found to be reduced by more than 2-fold[26] (lane 8 vs lane 5 in Figure 5). The methylation of Npl3p in the wild-type allele was essentially unaffected (lanes 1 to 4 in Figure 5). As a control, the protein expression levels of Npl3p in both wild-type and

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(26) The extent of methylation was estimated by the sum of all pixels in methylation bands (top panel) divided by the sum of all pixels in the corresponding protein spot (lower panel) using NIH Image software.

Table 3. Gene List for the Mutant *Rmt1-as1* Strain Treated with Compound **12** (|FC| > 2)[a]

category	gene name	fold change	primary role
cellular communication	CMK2	−2.1	calmodulin-dependent protein kinase (intracellular communication)
cellular organization	DHH1	2.1	putative RNA helicase of DEAD/DEAH box family (nuclear organization)
	HSP104	−2.5	heat shock protein 104 (stress response)
	YPS6	−2.1	GPI-anchored aspartic protease (extracellular/secretion proteins)
metabolism			
	GSC2	−3.4	catalytic component of 1,3-β-D-glucan synthase (C-compound and carbohydrate metabolism)
	MET1	−3.1	siroheme synthase (biosynthesis of vitamins, cofactors, and prosthetic groups)
	MET10	−2.6	subunit of assimilatory sulfite reductase (nitrogen and sulfur utilization)
	GAC1	−2.6	regulatory subunit for Glc7p (regulation of C-compound and carbohydrate utilization)
	MET2	−2.2	homoserine O-trans-acetylase (amino acid biosynthesis)
miscellaneous	CTR1	−2.3	high affinity copper transporter, probable integral membrane protein
	AFR1	−2.1	coordinates regulation of α-factor receptor signaling and induction of morphogenesis during conjugation
	PMC1	−2	putative vacuolar Ca2 + ATPase, ion transporters
	FRE1	−2	ferric (and cupric) reductase (homeostasis of metal ions)

[a] Only genes that have been annotated were listed.

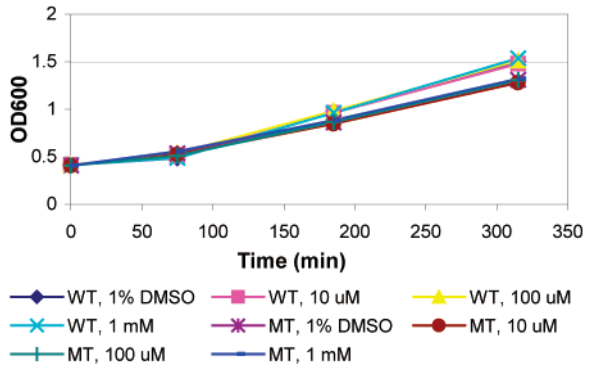

Figure 4. The yeast growth curve of wild-type or mutant alleles in the presence of varying concentrations of **15**.

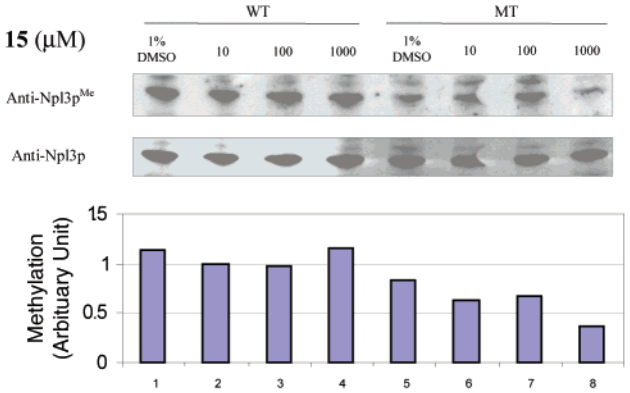

Figure 5. In vivo activity of **15** determined by western blot. Yeast strain BY4743 was used as the WT allele; Yeast strain W303 was used for the construction of the MT allele, *rmt1-as1*. The estimated extents of methylation are given in the bottom plot.

mutant alleles were shown not to alter upon treatment of **15** when a methylation independent anti-Npl3p antibody was used in the same western blot (lower panel Western in Figure 5).

In preliminary experiments the in vivo effects of the SAH analogues on the mutant alleles have been evaluated by mRNA expression profiling experiments with oligonucleotide microarray.[27] The *rmt1-as1* yeast cells were treated with 500 μM compound **12**, and the cells were harvested after 2 h. The mRNA expression was subject to gene chip analysis. Among 6200 yeast genes examined, we found only 30 genes (0.5%) having fold changes greater than 2.0 relative to untreated cells (Table 3). In addition to genes related to metabolism and cellular organization, a calmodulin-dependent protein kinase, *CMK2*, was found to be downregulated by 2.1-fold upon treatment. This finding suggests that protein phosphorylation might in some way be related to the protein methylation. Indeed, the phosphorylation of Ser411 of Npl3p by a kinase Sky1p has been reported recently, and this phosphorylation regulates nuclear import of Npl3p by modulating the interaction of RGG motif (the methylation site) with its nuclear import receptor Mtr10p.[28] Further profiling experiments with more potent methyltransferase inhibitors should give us a clearer picture on the Rmt1-mediated processes.

Discussion

For allele-specific substrates or inhibitors to be useful in gene family functional studies, the cross-reactivity between E and L* should be minimized (diagonal arrow in Figure 1). In the case of orthogonal substrates, the wild-type enzyme or its homologues should not accept the "bumped" substrates. At the same time, the mutant enzyme should preferentially accept the "bumped" substrate over the natural substrate. As expected, in our study the wild-type enzyme could not catalyze the methyltransfer reaction with the "bumped" SAM analogue **16**. The mutant enzyme could utilize natural SAM (with only 4-fold reduction in efficiency compared to wild-type enzyme), and could also utilize **16** with a k_{cat}/K_m roughly 450-fold lower than that of the wild-type enzyme for natural SAM (Table 1). This reduction of activity may also account for the slower growth rate observed for the mutant alleles than for the wild-type strain in the yeast growth experiment (Figure 4), as well as the reduced methylation of Npl3p (lane 5 vs lane 1 in Figure 5).

The naphthylmethyl-SAH analogue (**15**) was able to selectively inhibit the methyltransferase activity in enzymatic assays with a K_i of 5 μM (Table 2). In cell extracts, however, the inhibition of methylation of Npl3p by **15** was modest with an IC_{50} of 100 μM (Figure 3). Furthermore, the inhibitory activity of **15** was even lower when the mutant alleles were treated with **15** in cell culture and the methylation product was monitored by western blot ($IC_{50} \sim 100$ μM to 1 mM). Several factors may contribute to the observed low in vivo efficacy in yeast: (a) poor cell membrane permeability; (b) poor bioavailabilty; and (c) a rather long half-life (~4−5 h) for the substrate Npl3p used in this study. Similar poor membrane permeability has also been

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observed in the N^6-substituted ADP analogues.[29] Inhibitor designs based on alternative scaffolds, such as triazines and pyrimidines,[30] may yield more potent and selective methyltransferase inhibitors. Alternatively, the use of yeast lacking key drug pumps may increase the intracellular levels of inhibitors.

Preliminary profiling experiments with one of the compounds exemplified the potential utility of this type of selective inhibitor in the analysis of gene function. The downregulation of CMK2 kinase expression may indicate the existence of a regulatory mechanism in which the kinase activity and methyltransferase activity are interdependent. It was reported recently that Npl3p also undergoes phosphoration catalyzed by Sky1p kinase both in vitro and in vivo, and this phosphorylation affects Npl3p nucleocytoplasmic transport.[28] More potent methyltransferase inhibitors and experiments with synchronized yeast should provide additional insights into the cellular effects of the inhibitors.

The greatest advantage of the "bump-and-hole" approach is that an entire gene family can be studied in a parallel and systematic manner. Once a potent small-molecule inhibitor is identified, it can be quickly adapted to the study of all family members. For example, a "bumped" version of a very potent kinase inhibitor, 4-amino-1-*tert*-butyl-3-(*p*-methylphenyl)-pyrazolo[3,4-*d*]pyrimidine (PP-1), has been successfully applied in the functional analysis of a variety of tyrosine kinases.[3]

Conclusions

Many proteins are methylated in vivo including histone H4[31] and STAT1.[32] The identification of new methyltransferase substrates has proven important in the elucidation of the role of protein methylation in various processes. We have demonstrated that an orthogonal methyltransferase/inhibitor pair can be designed through the synergistic use of protein engineering and ligand design. The designed SAH analogue **15** can selectively inhibit the activity of mutant methyltransferase over the wild-type enzyme both in vitro and in vivo. Our approach should be generizable to all members of the protein methyltransferse family since all have a conserved Glu residue around the N^6 position of the adenosine ring in the active site. An orthogonal SAM cofactor (**16**) was also obtained with the same approach. This naphthylmethyl-SAM (**16**) is a selective substrate for the mutant methyltransferase. We have now begun to use the selective substrate and inhibitors described here to identify specific Rmt1 substrates in vivo.

Experimental Section

All chemicals were purchased from Sigma/Aldrich (St. Louis, MO) and used without further purification. 6-Chloroadenosine was purchased from General Intermediates of Canada (Edmonton, Alberta). The SAH analogues were synthesized according to ref 18 and the SAM analogue **16** was synthesized according to ref 19. All final compounds were purified by reverse phase preparative HPLC (C_{18} column, 10−90% acetonitrile/H_2O linear gradient in 30 min, 0.1% TFA added) and characterized by 400 MHz Bruker NMR and ES-MS.

***N*⁶-Benzyl-*S*-adenosylhomocysteine** (**12**): white powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.39 (m, 2H), 8.22 (s, 1H), 7.60 (s, b, 2H), 7.31 (m, 4H), 7.21 (m, 1H), 5.90 (d, J = 2.9 Hz, 1H), 5.52 (s, 1H), 5.44 (s, b, 1H), 4.76 (s, 1H), 4.71(s, 1H), 4.15 (s, 1H), 4.02 (s, 1H), 2.93 (m, 2H), 2.80 (m, 2H), 1.99 (m, 1H), 1.80 (m, 1H); ES-MS calcd for $C_{21}H_{27}N_6O_5S$ $[M + H]^+$ m/z 475.2, found 475.2.

***N*⁶-Naphthylmethyl-*S*-adenosylhomocysteine** (**15**): white powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.45 (s, b, 1H), 8.38 (s, 1H), 8.25 (m, 4H), 8.01 (t, J = 8.1 Hz, 1H), 7.95 (dd, J = 7.4, 2.0 Hz, 1H), 7.82 (d, J = 9.1 Hz, 1H), 7.68−7.52 (m, 4H), 7.46−7.41 (m, 2H), 5.91 (d, J = 5.7 Hz, 1H), 5.14 (s, 1H), 4.78 (s, 1H), 4.55 (m, 1H), 4.16 (m, 1H), 4.04−3.97 (m, 2H), 2.94 (dd, J = 13.8, 5.8 Hz, 1H), 2.83 (dd, J = 13.7, 7.5 Hz, 1H), 2.65 (m, 2H), 2.06−1.91 (m, 1H); ES-MS calcd for $C_{25}H_{29}N_6O_5S$ $[M + H]^+$ m/z 525.2, found 525.2.

***N*⁶-Benzyl-*S*-adenosylmethionine·TFA salt** (**16**): white powder; ^{1}H NMR (400 MHz, D_2O) δ 8.33 (s, 2H), 7.35 (m, 5H), 6.09 (d, J = 4.0 Hz, 1H), 4.70 (m, 2H), 4.54−4.46 (m, 2H), 3.96−3.77 (m, 4H), 3.62−3.57 (m, 1H), 3.40−3.37(m, 1H), 2.92 (s, 3H), 2.31−2.23 (m, 2H); ES-MS calcd for $C_{22}H_{29}N_6O_5S$ $[M]^+$ m/z 489.2, found 489.2.

Construction and Expression of GST-Rmt1, GST-Rmt1-E117G. The Glu^{117}→Gly mutant was generated by the QuickChange site-directed mutagenesis method (Stratagene, La Lolla, CA) with a pGEX-Rmt1 construct (a gift from Dr. Steve Clarke, UCLA) according to the manufacturer's instruction with the following primers: 5′-CCTTGCTAAGAGGCAAGTTGGSGGACGTTCATTTACCCTTTCC-3′ and 5′-GGAAAGGGTAAATGAACGTCCSCCAACTTGCC-TCTTAGCAAGG-3′. The resulting mutant plasmid was confirmed by DNA sequencing. The GST fusion proteins were expressed in *Escherichia Coli* BL21cells (Stratagene, La Lolla, CA) upon induction with a final concentration of 1.0 mM isopropyl-β-D-thiogalactopyranoside. Cells were washed and resuspended in 10 mL of lysis buffer (50 mM K-HEPES, pH 7.4; 250 mM KCl; 1% NP40; 1 mM EDTA; 1 mM DTT) and lysed by four 30 s sonicator pulses (40%) on ice with a dismembraner (Fisher Scientific). The resulting lysate was centrifuged at 23 000 g for 10 min at 4 °C. The fusion proteins were then purified from soluble extracts by binding to glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Proteins were eluted with a 10 mM glutathione elution buffer (50 mM Tris, 50 mM NaCl, pH 7.5). The protein concentrations were determined with use of the BioRad protein assay solution.

Kinetic Analysis. For kinetic analysis, 5 μL of R1 peptide (H-GGFGGRGGFG-NH_2, 1 mM), 10 μL of 10X assay buffer (25 mM Tris-HCl, 1 mM sodium EDTA, 1 mM sodium EGTA, pH 7.5), 83.5 μL of H_2O, and 1 μL of varying concentration SAM (final 0.2−10 μM) were mixed well and 0.5 μL of Rmt1 solution (0.213 μg) was added to the mixture. The reactions were incubated at 30 °C for 30 min, and then quenched by adding 40 μL of 1% TFA/H_2O. Aliquots of 10 μL sample solution were injected sequentially into an electrospray LC-MS instrument (Agilent Technologies). The mass peaks (m/z) of 441.2 ± 0.2 $(M - CH_3 + H)^{2+}$ (product) and 434.2 ± 0.2 $(M + H)^{2+}$ (starting material) were integrated to give ion intensities. The relative methylation velocity was defined as eq 1 and measured by following

$$v^{\mathrm{rel}} = \frac{\mathrm{d}\left(\dfrac{A_{(M-CH_3+H)^{2+}}}{A_{(M-CH_3+H)^{2+}} + A_{(M+H)^{2+}}}\right)}{\mathrm{d}t} \qquad (1)$$

the product formation over 12 min reaction courses. The reaction velocities were plotted against the cofactor concentration.

The values of $v_{\mathrm{rel,max}}$ and K were derived through fitting the curves with eq 2. The k_{cat} values were derived from eq 3. For inhibition studies,

$$\frac{V}{V_{\max}} = \frac{[S]}{K_{\mathrm{m}} + [S]} \qquad (2)$$

$$k_{\mathrm{cat}} = \frac{V_{\max}}{[E]_t} \qquad (3)$$

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1 μL of R1 peptide (1 mM), 1 μL of SAM (200 μM), 2 μL of 10X assay buffer, 14 μL of H_2O, and 1 μL of varying concentrations of inhibitor (100 nM to 10 mM) were mixed well and 1 μL of Rmt1 solution (0.425 μg) was added to the mixture. Methylation was monitored in the same way as in the kinetic analysis. The IC_{50} values for each compound were determined by examining the concentration-dependent inhibition curves. The K_i was calculated according to eq 4,

$$K_i = \frac{IC_{50}}{1 + [S]/K_m} \tag{4}$$

where [S] is the concentration of SAM, and K_m is the Michaelis−Menten constant for either wild-type or mutant enzyme.

Cell Extracts Methylation Assay. The Rmt1Δ yeast cells (Research Genetics, Huntsville, AL) were cultured in 250 mL of YPD media and harvested at OD = 2.4. The cells were spun down and the pellets were collected. The cells were lysed by using a standard glass bead method (Current Protocol in Molecular Biology 13.13.4). The supernatants were collected and the protein concentrations were determined by using a BioRad protein assay solution. Ten microliters of yeast extracts (57 μg) were incubated in 4.0 μL of ^{14}C-SAM (0.1 μCi, Amersham Pharmacia Biotech), 2.0 μL of Rmt1 (0.85 μg) or mutant enzyme (0.72 μg), 1 μL of varying concentration **15** (final 10−1000 μM) or DMSO control, and a buffer of 25 mM Tris-HCl, 1 mM sodium EDTA, and 1 mM sodium EGTA at pH 7.5 in a final volume of 25 μL. After 30 min at 30 °C, the reactions were stopped by adding 5 μL of 6X SDS-gel electrophoresis sample buffers. The samples were then separated by SDS-gel electrophoresis (12% acrylamide) and the gel fluorographed after 6 days of exposure at −80 °C. The ^{14}C intensities at the fluorographic bands of 55 KDa corresponding to Npl3p were integrated to give rise to the specific methylation extents of the cell extracts.

Construction of Yeast Rmt1-E117G Mutant Allele. The RMT1-E117G was subcloned into the integration plasmid pRS306 through the *Sal*I and *Not*I sites. The plasmid was linearized by using the *Swa*I restriction enzyme and transformed into yeast strain W303. The transformants were plated on a synthetic dropout (SD)-Ura3 plate. Single resistant colonies were picked and further selected on 5-FOA plates for the presence of a single copy *RMT1* gene. The strains that carry RMT1-E117G alleles were identified by sequencing the PCR products of the genomic DNA, using primers with identical sequences to the *RMT1* gene up- and downstream regions.

Western Blots. The wild-type (BY4743, Research Genetics, Huntsville, AL) and mutant yeast cells (W303, Mat a, bar1:hisG) were cultured in YPD media overnight. The cultures were then diluted into 4 portions for each strain (∼5 mL culture each). The cells were treated with either 50 μL of DMSO or compound **15** at varying concentrations. The cells were harvested after 5 h, and lysed by using a standard glass bead method. The supernatants were collected and the protein concentrations were determined by using a BioRad protein assay solution. Proteins (5 μg) were loaded onto a Tris-Glycine gel (12%, Invitrogen) and separated by SDS-PAGE. The proteins were then transferred onto a nitrocellulose membrane. The western blot detection was carried out by using the ECL kit (Amersham Pharmacia Biotech). For the methyl-specific antibodies, 1:250 ratio was used followed by 1:1000 anti-mouse HRP secondary antibodies. The antibodies were stripped according to the ECL instruction. To probe with anti-Npl3p antibodies, a 1:1000 ratio was used followed by 1:1000 anti-rabbit HRP secondary antibodies.

Acknowledgment. We thank Dr. X. Cheng for the coordinates of the PRMT3 structure, Dr. M. Swanson for the methyl-specific antibody 1E4, Dr. X. Fu for the anti-Npl3p antibody, and Dr. S. Clarke for the pGEX-Rmt1 plasmid. Q.L. is supported in part by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship, DRG-1612.

Note Added after ASAP: There were errors in Scheme 1 in the version posted ASAP November 1, 2001; the corrected version was posted November 9, 2001.

Supporting Information Available: Details of kinetics determination and inhibition assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA011423J