

## Small Molecule Regulators of Protein Arginine Methyltransferases\*

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**Here we report the identification of small molecules that specifically inhibit protein arginine *N*-methyltransferase (PRMT) activity. PRMTs are a family of proteins that either monomethylate or dimethylate the guanidino nitrogen atoms of arginine side chains. This common post-translational modification is implicated in protein trafficking, signal transduction, and transcriptional regulation. Most methyltransferases use the methyl donor, *S*-adenosyl-*L*-methionine (AdoMet), as a cofactor. Current methyltransferase inhibitors display limited specificity, indiscriminately targeting all enzymes that use AdoMet. In this screen we have identified a primary compound, AMI-1, that specifically inhibits arginine, but not lysine, methyltransferase activity *in vitro* and does not compete for the AdoMet binding site. Furthermore, AMI-1 prevents *in vivo* arginine methylation of cellular proteins and can modulate nuclear receptor-regulated transcription from estrogen and androgen response elements, thus operating as a brake on certain hormone actions.**

Protein arginine *N*-methyltransferases (PRMTs)<sup>1</sup> have been implicated in a variety of processes, including nuclear

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<sup>1</sup> The abbreviations used are: PRMT, protein arginine *N*-methyltransferase; aDMA, asymmetric  $\omega$ -*N*<sup>G</sup>,*N*<sup>G</sup>-dimethylarginine; sDMA, symmetric  $\omega$ -*N*<sup>G</sup>,*N*<sup>G</sup>-dimethylarginine; CARM1, coactivator-associated arginine methyltransferase 1; GAR, the glycine- and arginine-rich N-terminal region of fibrillarlin; hnRNP, heterogeneous nuclear ribonucleoprotein; snRNP, small nuclear ribonucleoprotein; PABP1, poly(A)-binding protein 1; TARPP, thymocyte cyclic-AMP regulated phosphoprotein; CBP, CREB (cAMP-response element-binding protein)-binding protein; SH3, Src homology 3; SMN, survival of motor neurons protein; AdoMet, *S*-adenosyl-*L*-methionine; AdoHcy, *S*-adenosyl-*L*-homocysteine; GST, glutathione *S*-transferase; AdOx, adenosine dialdehyde; SET domain, named after three *Drosophila* proteins that harbor this domain, *Su*(var), *Enhancer-of-zeste* and *Trithorax*; AMI, arginine methyltransferase inhibitor; AMA, arginine methyltransferase activator; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TK, thymidine kinase; AR, androgen receptor; ARE, androgen response element; CMV, cytomegalovirus; ERE, estrogen response element; HAT, histone acetyltransferase.

receptor-regulated transcription and protein trafficking (1). The methylation of arginine residues is catalyzed by Type I enzymes that facilitate the formation of asymmetric  $\omega$ -*N*<sup>G</sup>,*N*<sup>G</sup>-dimethylarginine (aDMA) residues, and by Type II enzymes that generate symmetric  $\omega$ -*N*<sup>G</sup>,*N*<sup>G</sup>-dimethylarginine (sDMA) residues (2). The cloning of the yeast Type I arginine methyltransferase enzyme (3, 4), Hmt1p (also known as Rmt1p), provided the molecular framework for the identification of six homologues in mammals (PRMT1–6). Currently, known mammalian Type I enzymes include PRMT1 (5, 6), the zinc finger-containing enzyme PRMT3 (7), the coactivator-associated arginine methyltransferase PRMT4/CARM1 (8), and the nuclear enzyme PRMT6 (9). The only mammalian Type II PRMT identified to date is the Janus kinase-binding protein Janus kinase-binding protein 1/PRMT5 (10, 11). Activity for the SH3 domain-containing arginine methyltransferase, PRMT2, has yet to be clearly demonstrated.

PRMTs target a wide array of different proteins for post-translational modification. The majority of aDMA residues occur within glycine- and arginine-rich (GAR) domains (12). Proteins with methylated GAR domains include Sam68, hnRNP K, hnRNP U, ILF3, FUS, and PABPN1 (13–16). PRMT4/CARM1 displays more specificity than other Type 1 enzymes and does not methylate GAR domains. PRMT4 substrates include CBP/p300, PABP1, TARPP, histone H3, and HuR (8, 16–20). Known substrates that contain sDMA (Type II PRMT targets) are myelin basic protein (21) and Sm ribonucleoproteins D1 and D3 (10, 22, 23).

Work in yeast unveiled the first identified biological role of arginine methylation: nuclear cytoplasmic shuttling of the hnRNP protein Npl3p requires Hmt1p activity (24). Recently, it was shown that protein-protein interactions are regulated by arginine methylation. The asymmetric dimethylation of arginine residues flanking proline-rich motifs can inhibit the binding of SH3 domains *in vitro* but does not interfere with the binding of the WW domains (a domain with two conserved tryptophans that binds proline-rich sequences) to the same motif (13). An opposite effect is seen with interactions between the survival of motor neurons protein (SMN) and the snRNP proteins SmD1 and SmD3, where symmetrical arginine dimethylation of the snRNP proteins enhances their binding to the Tudor domain of SMN (22).

Apart from regulating protein-protein interactions and protein trafficking, arginine methylation has also been implicated in the regulation of transcription. This became evident with the finding that the coactivator-associated arginine methyltransferase (PRMT4) binds the p160 family of nuclear hormone receptor coactivators. The presence of PRMT4 enhances transcriptional activation by nuclear receptors, possibly as a result

of the specific methylation of histone H3 by PRMT4 (8). PRMT1, like PRMT4, can also bind p160, and these two PRMTs have been shown to act in a synergistic manner to enhance reporter gene activation by nuclear receptors (25). The arginine methylation of histone H4 by PRMT1 facilitates the subsequent acetylation of H4 tails by p300 (26). In addition, PRMT4 can regulate the activity of p300/CBP (17, 18). Thus, lysine acetylation and arginine methylation work together to enhance nuclear receptor-regulated transcription.

Further attempts to elucidate the biological roles of arginine methylation have included the targeted disruption of the *Prmt1* and *Prmt4* genes in mice (20, 27) and the use of broad-spectrum inhibitors of methylation. Studies involving small molecule inhibitors have revealed that a loss of methylation can change the subcellular localization of methylated proteins (28, 29), prevent nerve growth factor-induced neuronal differentiation of PC12 cells (30–32), and inhibit the membrane ruffling seen within seconds after nerve growth factor is added to PC12 cells (33). However, interpretation of these findings is impaired by the global nature of methylation inhibition, which makes it difficult to assign specific cellular functions to a distinct methylation event. The compounds used in these studies are of two types: first, small molecules that inhibit *S*-adenosyl-L-homocysteine (AdoHcy) hydrolase, like adenosine dialdehyde (AdOx), result in a marked intracellular accumulation of AdoHcy. Most methylation reactions are affected through feedback inhibition by elevated levels of AdoHcy. Second, analogues of AdoMet like sinefungin and methylthioadenosine also function as inhibitors of methylation. The inhibition of AdoHcy hydrolase by small molecules and the use of AdoMet analogues can affect cellular methylation of phospholipids, proteins, DNA, and RNA. Recently, analogues of AdoMet were used to selectively inhibit a mutant form of the yeast protein methyltransferase, Hmt1p, using a “bump-and-hole” approach (34).

To date, no specific chemical modulators of endogenous protein methyltransferases or of specific arginine methyltransferases have been identified. Here we identify such molecules that will help elucidate the biological role of arginine methylation and perhaps serve as lead compounds for future drug development.

#### EXPERIMENTAL PROCEDURES

**Antibodies and Plasmids**—The  $\alpha$ GFP antibody was purchased (Clontech, Palo Alto, CA). The characterization of the 1E4 monoclonal antibody has been described previously (35). The  $\alpha$ Sam68 and  $\alpha$ PRMT1 antibodies were a gift from Stéphane Richard. All GST fusion proteins were subcloned in pGEX6P-1 (Amersham Biosciences), which allows for the induced production of recombinant proteins fused to the C-terminal end of glutathione *S*-transferase. GST-PABP1 (16) and GST-SmB' (36) have been described. GST-PRMT1, GST-PRMT3, GST-PRMT4, and GST-PRMT6 have been described previously (9). GST-Suv39H1 and GST-Suv39H2 were a gift from Thomas Jenuwein and were described previously (37). GST-SET7 and GST-DOT1 were gifts from Yi Zhang (38). GST-Npl3 and GST-Hmt1p were a gift from Pam Silver and have been described previously (39). For the GFP-Npl3 fusion construct, full-length Npl3 from pGEX-Npl3 was amplified by PCR primer 1 (5'-GTGGGATCCACCATGTCTGAAGCTCAAGA-3') and primer 2 (5'-AGAGGATCCAACTGGTTGTGATCTTTCACG-3'). The 5' primer introduced a mammalian “Kozak” sequence (CACC) just upstream of the initiator ATG. The fragments were subcloned in pd2EGFP-N1 (Clontech) to generate a N-terminal fusion of Npl3. Histones H3 and H4 (calf thymus) were purchased from Roche Applied Science.

**Compound Screening**—The 1E4 antibody was used to establish an ELISA-based high throughput screen for small molecule inhibitors of arginine methylation. We used a 384-well white high binding plates (Costar) coated with 100 ng of GST-Npl3 (the substrate). The plates were blocked with 5% BSA and incubated with GST-Hmt1p (500 ng of enzyme). Small molecules were then robotically transferred to individual wells to a final concentration of 10–15  $\mu$ M. Finally, the enzyme reaction was initiated by the addition of the methyl donor, AdoMet (10  $\mu$ M). The reaction was incubated for 1 h at room temperature. The wells

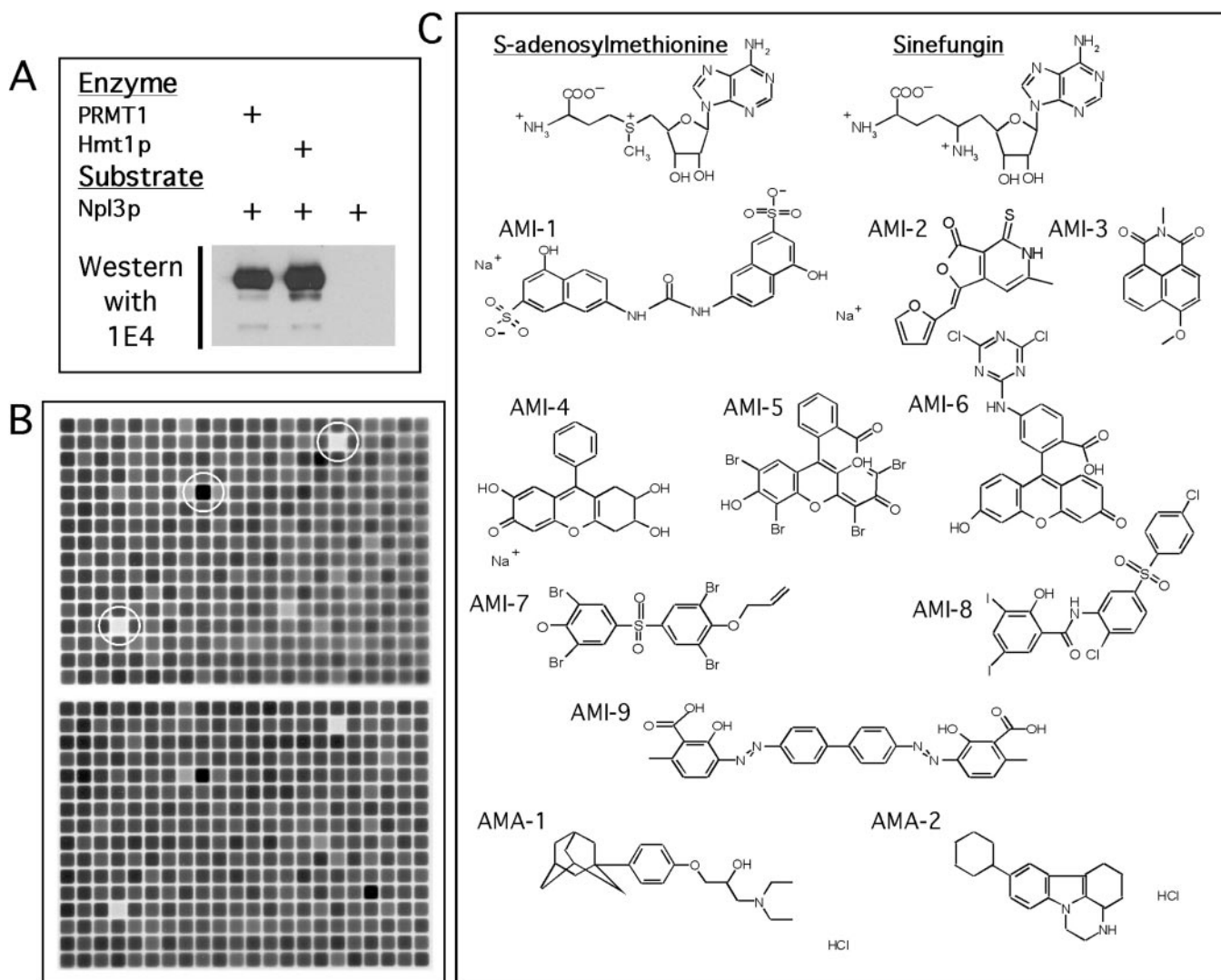
were then washed twice with TBST (25 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20) and blocked in TBST containing 2% BSA for 30 min. A mix of methyl-specific primary antibody (1E4 at 1:1000) and secondary antibody ( $\alpha$ mouse-peroxidase at 1:10000) was added to each well and incubated at room temperature for an additional hour. The wells were then washed three times with TBST and subjected to enhanced chemiluminescence. The chemiluminescent signal from each plate was detected by autoradiography and a Wallac multilabel counter. 9000 individual compounds from a diverse collection of synthetic chemicals (DIVETSet from Chembridge, San Diego, CA) were screened. This screen was performed in duplicate, and potential “hits” were those compounds that displayed at least a 60% reduction (in duplicate) in chemiluminescent signal as assayed by a Wallac multilabel counter. The compounds were solubilized in Me<sub>2</sub>SO to generate stock solutions. The molecular mass of AMI-1 (the primary compound) and AMI-5 were confirmed by mass spectrometric analysis, thus establishing that the structures are correct.

**Colorimetric Assay to Establish IC<sub>50</sub> Values**—A colorimetric assay was established to determine the IC<sub>50</sub> of compounds. Briefly, 500 ng of GST-Npl3 in 50  $\mu$ l of TBS was added to each well of an EIA/RIA 96-well plate (Corning Inc., Corning, NY). After incubation at 4 °C overnight, the plate was rinsed with TBST and once with 20 mM Tris buffer (pH 8.0). The compounds were added to the GST-Npl3-coated plates. After a 15-min incubation, 200 ng of PRMT1 or Hmt1p and 1  $\mu$ l of AdoMet (from a 5 mM stock) were added into each well in a final volume of 50  $\mu$ l of 20 mM Tris buffer (pH 8.0) and incubated at 30 °C for 1 h. The plate was washed with TBST and then blocked with 5% BSA in TBST buffer for 2 h at room temperature. Anti-Npl3 antibody 1E4 (1:5,000) was then added to each well and shaken for 2 h at room temperature. The wells were washed with TBST. Secondary antibody ( $\alpha$ mouse-peroxidase at 1:10,000) was then added to each well, and the mixture was incubated for 1 h at room temperature. The wells were again rinsed with TBST. The peroxidase substrate solution, ABTS, was added to each well, and the mixture was incubated for 1 h. The absorbance was measured at 415 nm with a plate reader (Bio-Rad). IC<sub>50</sub> for compounds was calculated by linear regression analysis of percentage inhibition.

**In Vitro Methylation Assay**—All methylation reactions were carried out in the presence of *S*-adenosyl-L-[methyl-<sup>3</sup>H]methionine (<sup>3</sup>H]AdoMet, 79 Ci/mmol from a 12.6  $\mu$ M stock solution in dilute HCl/ethanol 9:1, pH 2.0–2.5, Amersham Biosciences) and PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). The reactions were performed in a 30- $\mu$ l volume in the presence of 5  $\mu$ g of the small molecule inhibitors, this translates into the following concentration of each compound: AMI-1, 303  $\mu$ M; AMI-2, 642  $\mu$ M; AMI-3, 690  $\mu$ M; AMI-4, 520  $\mu$ M; AMI-5, 257  $\mu$ M; AMI-6, 336  $\mu$ M; AMI-7, 265  $\mu$ M; AMI-8, 247  $\mu$ M; AMI-9, 325  $\mu$ M; AMA-1, 439  $\mu$ M; AMA-2, 503  $\mu$ M; and sinefungin, 437  $\mu$ M. Additional information pertaining to individual reaction conditions is described in each of the figure legends. The samples were separated on a 10% SDS-PAGE, transferred to a PVDF membrane, sprayed with Enhance<sup>TM</sup> (PerkinElmer Life Sciences, Boston, MA), and exposed to film overnight. Band intensities were calculated using a Kodak Image Station 440 and 1D Image Analysis Software (Eastman Kodak Co.).

**In Vivo Methylation Assay**—HeLa cells were labeled using a previously described *in vivo* methylation assay (40). The cells were lysed in a “mild” buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM Tris-HCl, pH 7.5), and immunoprecipitations were performed with  $\alpha$ Sam68 antibodies. Samples were then subjected to fluorography.

**Luciferase Assays and Transient MCF7 Transfections**—ERE-TK-firefly has a single vitellogenin estrogen response element containing a basal thymidine kinase (TK) promoter linked to firefly luciferase (41). ARE-firefly and AR were a gift from Mickey Hu (42). phRL-CMV has humanized *Renilla* luciferase driven by CMV promoter (Promega, Madison, WI). MCF7s were maintained in phenol red-free Dulbecco's modified Eagle's medium supplemented with 5% charcoal stripped fetal bovine serum. Approximately 20 h before transfection, 40,000 cells were seeded into each well of 12-well culture dishes. The cells in each well were transfected with FuGENE6 transfection reagent (Roche Applied Science) according to the manufacturer's protocol. For each transfection, 5 ng of phRL-CMV and 295 ng of ERE-TK-Luc were used for estrogen response and 5 ng of phRL-CMV, 148 ng of AR, and 148 ng of ARE were used for androgen response. After 4 h of transfection, cells were treated with either 10 nM estradiol (Sigma) or 10 nM R1881 (PerkinElmer Life Sciences) and the indicated amounts of AMI-1. The indicated amounts of GFP-PRMT1 and pCAGGS-CARM1 were used for the rescue experiments. In all cases DNA in each well was adjusted to 500 ng with Bluescript plasmid. The reporter was constitutively active under these conditions. After 42–44 h, the cells were washed twice with



**FIG. 1. High throughput screen for compounds that affect arginine methyltransferase activity and the chemical structure of hits.** **A**, recombinant Hmt1p and PRMT1 generate a methylation-dependent epitope on Npl3p that is recognized by the monoclonal antibody, 1E4. GST-Npl3 (0.1  $\mu$ g) was incubated, in a final volume of 20  $\mu$ l of PBS containing 10  $\mu$ M AdoMet, with either GST-Hmt1p (0.5  $\mu$ g), GST-PRMT1 (0.5  $\mu$ g), or no enzyme. The samples were separated on a 10% SDS-PAGE, transferred to a PVDF membrane, and analyzed by Western with 1E4. **B**, the high throughput screen was performed in duplicate. GST-Hmt1p (0.5  $\mu$ g) and GST-Npl3 (0.1  $\mu$ g) were added to each well in a volume of 20  $\mu$ l of PBS. Next, small molecules were robotically transferred to individual wells to a final concentration of 10–15  $\mu$ M, and the enzyme reaction was initiated by the addition of the methyl donor, 10  $\mu$ M AdoMet. Following the reaction, the wells were incubated with a mixture of primary antibody (1E4 at 1:1,000) and secondary antibody ( $\alpha$ mouse-HRP at 1:10,000) and washed with TBST three times, and the horseradish peroxidase signal was detected with the ECL kit (Amersham Biosciences). Autoradiography film was placed over the ELISA plate for 30 s and then developed. The autoradiograph of one set of 52 plates screened is shown. Circles mark the position of “hits” on the plate. The primary identification of two inhibitors and one activator is shown. **C**, chemical structure of AdoMet, sinefungin, the nine most potent inhibitors (AMIs), and two activators (AMAs) of the Hmt1p arginine methyltransferase.

PBS and harvested to perform luciferase assay using the Dual Luciferase Assay System (Promega).

**Photoaffinity Competition Labeling of Methyltransferase Enzymes**—UV cross-linking of *S*-adenosyl-L-[methyl- $^3$ H]methionine to Hmt1p was performed as previously described (43, 44). A CL-1000 UV cross-linker was used (UVP, Upland, CA). GST-Hmt1p (10  $\mu$ g) without any competitor or with 200  $\mu$ M sinefungin, 200  $\mu$ M AdOx, 200  $\mu$ M AMI-1, and AMI-5, respectively, was exposed to UV light (254 nm) at a distance of 1 cm for 30 min at 4  $^{\circ}$ C in the presence of 3.2  $\mu$ M [ $^3$ H]AdoMet (79 Ci/mmol, from a 12.6  $\mu$ M stock solution in dilute HCl/ethanol 9:1, pH 2.0–2.5, Amersham Biosciences) and 5 mM dithiothreitol in a total volume of 50  $\mu$ l of PBS. After UV cross-linking, samples were run on SDS-PAGE and subjected to fluorography.

## RESULTS

**Chemical Screening for Arginine Methyltransferase Inhibitors**—The *Saccharomyces cerevisiae* RNA-binding protein, Npl3p, is arginine-methylated *in vivo* (35). A monoclonal antibody, 1E4, was raised against Npl3p (45), and it was later

found that the epitope recognized by 1E4 is generated by an arginine methylation event (35). The 1E4 antibody does not recognize bacterial recombinant Npl3p. However, recombinant Npl3p that is methylated *in vitro* either with the yeast (Hmt1p) or the human (PRMT1) recombinant arginine methyltransferases generates the 1E4-binding epitope (Fig. 1A). These properties make the 1E4 antibody superlative for use in a small molecule screen for inhibitors of this post-translational modification.

Using the 1E4 antibody, we established an ELISA-based method that was suitable for a high throughput small molecule screen for inhibitors of arginine methyltransferase activity. We used a 384-well plate format to immobilize GST-Npl3. The plates were then blocked with BSA and incubated with GST-Hmt1p. Small molecules were robotically arrayed into individual wells. Finally, the enzyme reaction was initiated by the addition of the methyl donor, AdoMet. As the primary read-out

TABLE I  
*IC*<sub>50</sub> of inhibitors and properties of activators

Chembridge #	AMI-	Molecular weight	<i>IC</i> <sub>50</sub>	
			For PRMT1	For Hmt1p
<i>μM</i>				
Inhibitors				
Sinefungin		381.40	1.63	3.04
5255879	1	548.45	8.81	3.03
5112759	2	259.28	11.18	3.37
5255910	3	241.25	16.29	4.25
5108509	4	320.30	0.19	0.15
5122150	5	647.90	1.41	0.78
5135283	6	495.28	5.11	6.88
5157220	7	627.90	1.61	1.50
5175464	8	674.08	1.80	0.43
5193079	9	512.52	0.28	0.26
Chembridge #	AMA-	Molecular weight	Activity of PRMT1	Activity of Hmt1
%				
Activators				
5217339	1	379.23	326.6	298.0
5229971	2	330.89	245.0	285.0

in the screen for arginine methyltransferase inhibitors we used the methylation-sensitive antibody, 1E4. 1E4 binding was detected using peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence. The chemiluminescent signal from each plate was detected by autoradiography (Fig. 1B) and a Wallac multilabel counter. This screen was performed in duplicate, on 9000 individual compounds from a diverse collection of synthetic chemicals (ChemBridge), with very reproducible results (Fig. 1B). In this manner, we identified nine compounds that inhibited the methylation of Npl3p by Hmt1p (Fig. 1C). We have called these compounds AMIs for arginine methyltransferase inhibitors. As would be expected from this type of screen, we found analogues of AdoMet (AMI-2) as well as compounds that are structurally related (AMIs-4, -5, and -6). Unexpectedly, we identified two compounds that enhance the *in vitro* methylation of Npl3p by Hmt1p (Fig. 1, B and C), and we refer to these compounds as AMAs for arginine methyltransferase activators.

The *IC*<sub>50</sub> (the concentration of compound required to reduce the enzyme activity by 50%) values for the AMIs ranged from 0.15 to 7  $\mu$ M, and they were able to inhibit the mammalian orthologue of Hmt1p, PRMT1 (Table I). The activators stimulated methylation by both the yeast and the human enzymes up to 3-fold.

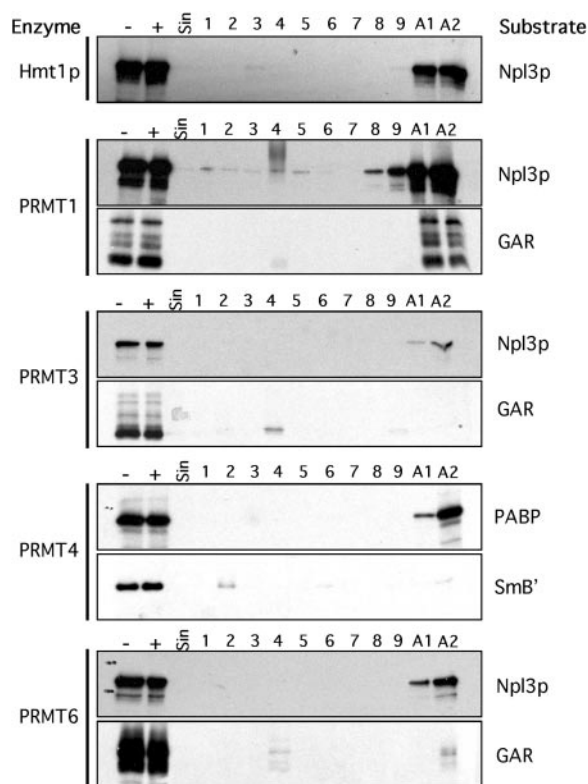
**AMI-1 Inhibits Arginine, but Not Lysine, Methyltransferases *in Vitro***—To investigate which of the AMIs to further analyze, we tested the specificity of these compounds on a panel of arginine methyltransferases. All the arginine methyltransferases (Hmt1p and PRMT1, -3, -4, and -6) that are active as GST fusion proteins were assayed against two different substrates each (Fig. 2). The nine AMIs inhibited all the tested PRMTs.

The major protein methylation sites within cells are not only on arginine residues but also on the side-chain nitrogens of the amino acid lysine (46). Mono-, di-, and trimethylation of lysine residues is catalyzed by a unique family of methyltransferases that harbor SET domains (47). The cofactor-binding region of the SET domain is structurally distinct from the AdoMet binding folds of arginine and DNA methyltransferases (48). The fact that the active domains of SET domain-containing proteins and arginine methyltransferases are dissimilar increases the possibility of identifying specific inhibitors. Thus, we tested the ability of the AMIs to compromise lysine methyltransferase function. The SET domain-containing proteins Suv39H1, Suv39H2, and SET7 were assayed in the presence of the AMIs

for their ability to methylate histones H3. In addition, the potency of the inhibitors was also assessed on the non-SET domain-containing lysine methyltransferase, DOT1 (49). AMI-2, -3, -4, -5, -7, -8, and -9 were able to inhibit both lysine and arginine methyltransferases (Fig. 3) or displayed a lack of specificity and are, therefore, of less interest to us than the PRMT-specific inhibitors. Compounds that inhibited the tested arginine methyltransferases but not the tested SET domain-containing enzymes (Fig. 3, AMI-1 and -6) were the focus of further investigation. In addition, AMI-5 was used as an example of a nonspecific small molecule inhibitor in the following experiments. AMI-5 inhibits the tested PRMT enzyme set (Figs. 2 and 3) and the lysine methyltransferases DOT1 and SET7, but not Suv39H1 and -2 enzymes (Fig. 3).

AMA-1 and AMA-2 were identified as activators of Hmt1p action in both the primary screen (Fig. 1B) and when using the colorimetric assay developed to assess the potency of the various compounds (Table I). In the *in vitro* methylation reaction, portrayed in Fig. 2 (*lanes A1* and *A2*), this effect is not seen, possibly due to the fact that less peptide substrate is coated on an ELISA plate than is present in a [<sup>3</sup>H]AdoMet driven *in vitro* reaction, or that more AdoMet is present in the first set of experiments (Fig. 1B and Table I). However, the activators do display some unique properties. Apart from enhancing Hmt1p and PRMT1 activity, AMA-1 inhibited PRMT3, -4, and -6 (Fig. 2, *lane A1*). With regard to the SET domain-containing proteins tested, AMA-1 and -2 inhibited Suv39H1 and Suv39H2, but not SET7 or DOT1 (Fig. 3). AMA-2 was able to inhibit the methylation of SmB' by PRMT4 (Fig. 2, *lane A2*) but not the methylation of PABP1 by the same enzyme. This phenomenon of substrate-specific inhibition is also seen with PRMT6. It should also be noted that AMI-2, -3, and -4 are not very effective inhibitors of PRMT4 methylation activity directed toward histone H3 (Fig. 3), but the same compounds effectively prevent the methylation of PABP and SmB' by PRMT4 (Fig. 2). The substrate-dependent nature of enzyme/inhibitor pairs likely reflects different ways in which the various substrates interact with the active site of the same PRMT.

**AMI-1 Inhibits Methylation of GFP-Npl3 and Cellular Proteins**—To determine whether the compounds that showed specific arginine methyltransferase inhibitory properties (AMI-1 and -6) were able to inhibit PRMT activity within a cellular context, we generated a fusion between the green fluorescence protein (GFP) and the yeast protein Npl3. We have established that mammalian PRMT1 can methylate Npl3 *in vitro* (Fig. 1A),

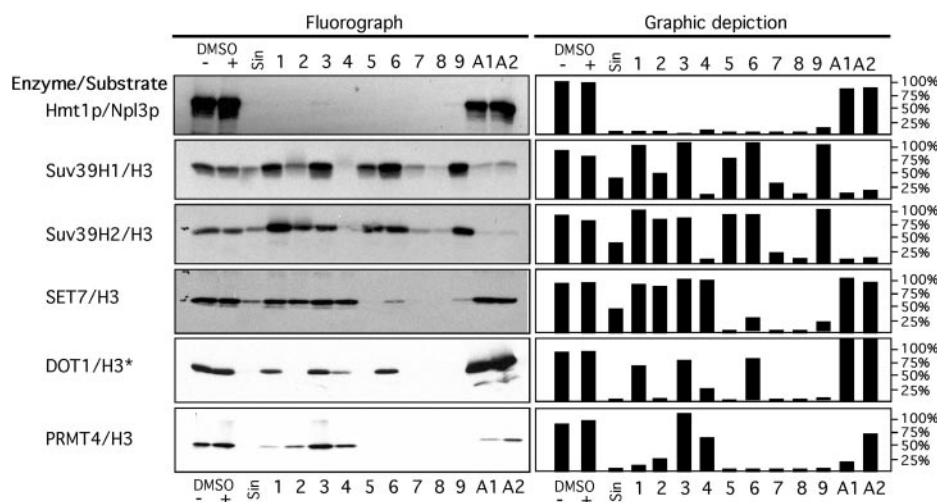


**FIG. 2. Inhibitory effects of the compounds on a broad set of arginine methyltransferases.** *In vitro* methylation reactions were performed with recombinant PRMTs and a set of different substrates, in the presence of the small molecules. GST-Hmt1p was incubated with GST-Npl3 as substrate. GST-PRMT1, -PRMT3, and -PRMT6 were incubated with GST-Npl3 and GST-GAR as substrates. GST-PRMT4 was incubated with GST-PABP and GST-SmB' as substrates. Substrates (0.5  $\mu$ g) were incubated with recombinant enzymes (0.2  $\mu$ g) in the presence of 0.5  $\mu$ M [ $^3$ H]AdoMet and the indicated small molecule (5  $\mu$ g) for 90 min at 30  $^{\circ}$ C in a final volume of 30  $\mu$ l of PBS. Reactions were separated on a 10% SDS-PAGE, transferred to a PVDF membrane, sprayed with Enhance<sup>TM</sup> (PerkinElmer Life Sciences) and exposed to film overnight. Reactions were performed in the presence (+) and absence (-) of Me<sub>2</sub>SO (the small molecule solvent) at 3.3% v/v. Sinefungin (*Sin*) is included as an AdoMet analogue. AMIs (lanes 1-9) and AMAs (lanes A1 and A2) are indicated.

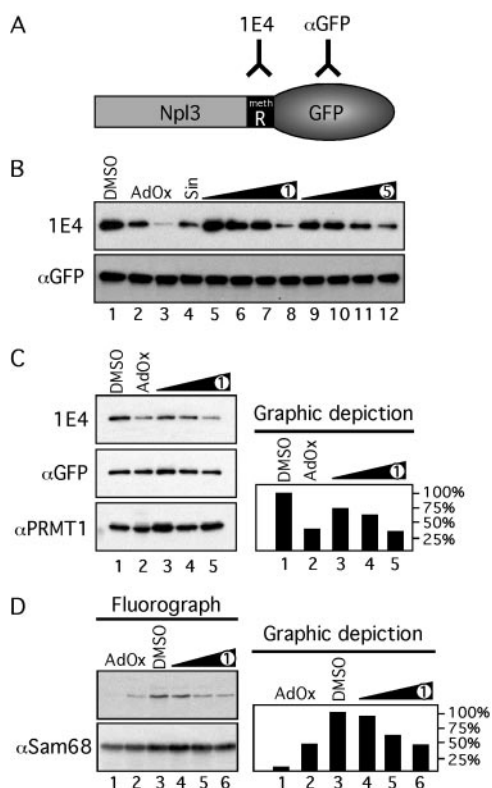
thus we reasoned that this reaction would also take place within a mammalian cell line. A destabilized GFP variant was used that displays rapid turnover rates. This shorter half-life makes destabilized variants suitable for use in quantitative reporter assays. The GFP-Npl3 was transiently transfected into HeLa cells and post-transfection the cells were treated for 24 h with 20-fold the IC<sub>50</sub> of AMI-1, -5, and -6. Because GFP and Npl3 are fused, the  $\alpha$ GFP antibody was used to establish equal loading and  $\alpha$ Npl3 antibody (1E4) acted as the methylation sensor (Fig. 4A). Thus, the relative degree of arginine methylation in the presence of the different inhibitors can be established. Using this assay system we demonstrated that AMI-1 and AMI-5 were able to inhibit methylation levels of GFP-Npl3 fusion, however AMI-6 exhibited a minimal effect on GFP-Npl3 fusion methylation levels (data not shown). We thus focused our attention on AMI-1 as a lead compound (AMI-5 is included in subsequent experiments as a control; it, however, also inhibits SET7 and DOT1 activity (Fig. 3)).

A concentration gradient of AMI-1 (2–10  $\mu$ M) and AMI-5 (0.2–2  $\mu$ M) was used to treat GFP-Npl3 transiently transfected HeLa cells for 24 h. The concentration ranges reflect the IC<sub>50</sub> values of the compounds. Total cell extracts were then subjected to Western analysis with  $\alpha$ GFP and 1E4 (methyl-sensitive  $\alpha$ Npl3) antibodies. Both AMI-1 and -5 inhibited the methylation of Npl3 within a cell (Fig. 4B). In addition, the inhibitors of global methylation, AdOx and Sinefungin, also reduced the methylation status of this reporter. It should be noted that this is not an assay for the cell permeability of the compounds, because the transfection agent (LipofectAMINE) may still be present on the cell surface and could facilitate small molecule uptake.

To determine if AMI-1 was indeed cell-permeable we generated a HeLa cell line that stably expressed GFP-Npl3. This cell line was treated with the arginine methylation inhibitor for 7 days. Over this period AMI-1 is not cytotoxic to HeLa cells even at 20-fold the IC<sub>50</sub> value (data not shown), here we used concentrations of 30  $\mu$ M ( $\approx 3.5 \times$  IC<sub>50</sub>), 60  $\mu$ M ( $\approx 7 \times$  IC<sub>50</sub>), and 120  $\mu$ M ( $\approx 14 \times$  IC<sub>50</sub>). Again, total cell extracts were then subjected to Western analysis with the 1E4 (methyl-sensitive  $\alpha$ Npl3) antibody (Fig. 4C, upper panel), after which the blot was

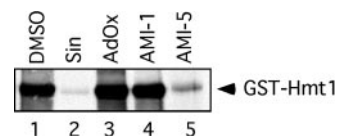


**FIG. 3. The effect of the compounds on SET domain mediated lysine methylation.** To determine the specificity of the small molecules, *in vitro* methylation reactions were performed with arginine and lysine methyltransferases. GST-Hmt1p was incubated with GST-Npl3 (0.5  $\mu$ g) as substrate. GST-Suv39H1, GST-Suv39H2, GST-SET7, and GST-PRMT4 were incubated with the same substrate, H3 (2.0  $\mu$ g). GST-DOT-1 was incubated with nucleosomes (0.2  $\mu$ g) as substrate. Substrates were incubated with recombinant enzymes (0.2  $\mu$ g) in the presence of 0.5  $\mu$ M [ $^3$ H]AdoMet and the indicated small molecule (5  $\mu$ g) for 90 min at 30  $^{\circ}$ C in a final volume of 30  $\mu$ l of PBS. Reactions were run on a 10% SDS-PAGE, transferred to a PVDF membrane, sprayed with Enhance<sup>TM</sup>, and exposed to film overnight. The fluorographs are shown in the left panel, and the quantification of the methylation levels is depicted in the right panel. Reactions were performed in the presence (+) and absence (-) of Me<sub>2</sub>SO at 3.3% v/v. Sinefungin (*Sin*) is included as an AdoMet analogue. AMIs (lanes 1-9) and AMAs (lanes A1 and A2) are indicated. H3\* indicates that DOT1 methylates histone H3 in the context of nucleosomes.



**FIG. 4. Effects of compounds on cellular arginine methyltransferase activity.** *A*, a depiction of the GFP-Npl3 fusion protein with the position of methylated region and the antibodies that recognize it. *B*, HeLa cells were grown in 12-well plates and then transiently transfected with d2GFP-Npl3. Three hours post-transfection, the cells were incubated with the indicated compounds for 24 h. The cells were lysed in RIPA buffer, and Western analysis was performed with either the 1E4 antibody (*top panel*) or  $\alpha$ GFP antibody (*bottom panel*). The effects of the compounds on GFP-Npl3 methylation status were established with the methyl-specific antibody, 1E4. The  $\alpha$ GFP antibody showed the protein levels of GFP-Npl3. Me<sub>2</sub>SO (0.25% v/v) was the vehicle for the compounds (*lane 1*). The concentrations of the compounds are 2  $\mu$ M AdOx (*lane 2*), 10  $\mu$ M AdOx (*lane 3*), 20  $\mu$ M sinefungin (*lane 4*), AMI-1 (2, 4, 6, and 10  $\mu$ M) (*lanes 5-8*), AMI-5 (0.2, 0.4, 0.8, and 2.0  $\mu$ M) (*lanes 9-12*). *C*, HeLa cells were stably transfected with GFP-Npl3. Cells were incubated with either AdOx or three different concentrations of AMI-1 for 7 days. Cell growth medium was replaced every day and supplemented the indicated compounds. Following treatment, cells were lysed in RIPA buffer and Western analysis was performed with either the 1E4 antibody (*top panel*) or  $\alpha$ GFP antibody (*middle panel*). The effects of the compounds on GFP-Npl3 methylation status was established with the methyl-specific antibody, 1E4. Quantification of the methylation levels of compound-treated samples, relative to vehicle-treated sample, is depicted in the *right panel*. The  $\alpha$ GFP antibody showed equal protein levels of GFP-Npl3. The  $\alpha$ PRMT1 antibody showed that the protein levels of PRMT1 are unaffected by AMI-1 (*bottom panel*). Me<sub>2</sub>SO (0.3% v/v) was the vehicle for the compounds (*lane 1*). The concentrations of the compounds are 20  $\mu$ M AdOx (*lane 2*), AMI-1 (30, 60, and 120  $\mu$ M) (*lanes 3-5*). *D*, *in vivo* methylation of Sam68 is inhibited by AMI-1. Methylated proteins were labeled *in vivo* in the presence of AdOx or AMI-1. Immunoprecipitations were performed with  $\alpha$ Sam68 antibodies. The <sup>3</sup>H-labeled proteins were visualized by fluorography (1-day exposure time) (*upper panel*), and the same membrane was subsequently immunoblotted with an  $\alpha$ Sam68 antibody (*lower panel*). Quantification of the methylation levels of compound-treated samples, relative to vehicle-treated sample, is depicted in the *right panel*. The Western analysis with the  $\alpha$ Sam68 antibody showed equal protein levels. Me<sub>2</sub>SO (0.3% v/v) was the vehicle for the compounds (*lane 3*). The concentrations of the compounds are AdOx (20 and 4  $\mu$ M, *lanes 1 and 2*), AMI-1 (30, 60, and 120  $\mu$ M, *lanes 4-6*).

striped and re-probed with an  $\alpha$ GFP antibody to confirm equal loading (Fig. 4C, *middle panel*). PRMT1 levels remain constant in the presence of increasing AMI-1 concentrations (Fig. 4C, *lower panel*). The methylation inhibitory properties of AMI-1 are graphically depicted in the *right panel* (Fig. 4C) by record-



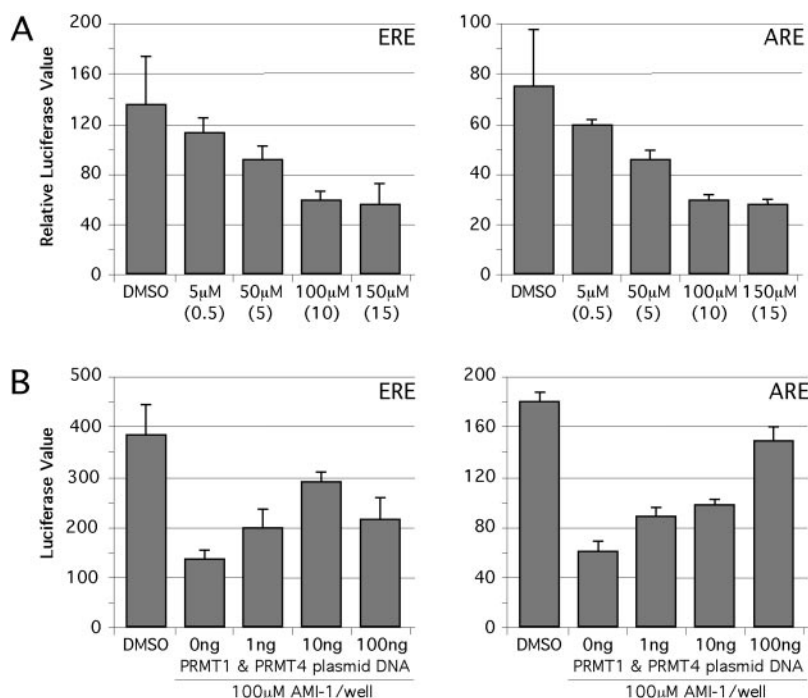
**FIG. 5. AMI-1 does not compete for AdoMet binding to Hmt1p.** GST-Hmt1p was UV cross-linked to [<sup>3</sup>H]AdoMet in the absence of any competitor (*lane 1*). Cross-linking in the presence of potential competitors was performed with: 200  $\mu$ M sinefungin (*lane 2*), 200  $\mu$ M AdOx (*lane 3*), 200  $\mu$ M AMI-1 (*lane 4*), and 200  $\mu$ M AMI-5 (*lane 5*). Samples were separated on a 10% SDS-PAGE, transferred to a PVDF membrane, sprayed with Enhance<sup>TM</sup>, and exposed to film overnight.

ing the intensity of each band in the compound-treated lanes, from the 1E4 Western, relative to the intensity of the vehicle-treated sample. In this experiment, as opposed to the transient transfection experiment, higher concentrations of AMI-1 were required before an effect on Npl3 methylation was seen. This could be due either to the fact that more compound enters the cell in the transient transfection experiment or that with longer exposures the cell can metabolize the compound to some degree. These data indicate the AMI-1 is cell-permeable and that it inhibits endogenous PRMT1-like activity.

To establish that AMI-1 can regulate the post-translational modification rates of endogenously methylated proteins, we chose to look at the well characterized PRMT1 substrate, Sam68 (14). The methylated protein pool was labeled with tritium in HeLa cells using an *in vivo* methylation assay described by Liu and Dreyfuss (40). Using this assay, methylated proteins were labeled by incubating cells with L-[methyl-<sup>3</sup>H]methionine in the presence of the protein synthesis inhibitor, cycloheximide. The labeling was performed in the presence of AMI-1 at concentrations of 30, 60, and 120  $\mu$ M. Sam68 was immunoprecipitated from these labeled cell extracts and subjected to fluorography (Fig. 4D, *upper panel*). The same membrane was immunoblotted with  $\alpha$ Sam68 antibodies to confirm equal loading (Fig. 4D, *lower panel*). The methylation inhibitory properties of AMI-1 toward Sam68 are graphically depicted in the *right panel* (Fig. 4D) by recording the intensity of each band in the compound-treated lanes from the fluorograph, relative to the intensity of the vehicle-treated sample. These results show that AMI-1 can inhibit the arginine methylation of the endogenously methylated protein, Sam68.

**AMI-1 Does Not Compete for the AdoMet Binding Site**—Recently, PRMT1, PRMT3, and RMT1 have been crystallized, and their core structures have proven very similar (39, 50, 51). The locations of the AdoMet binding site and the active site arginine-binding pocket were first inferred from the position of conserved sequence motifs and topological features (39, 51). It was then established with the crystal structure of PRMT1 together with the reaction product AdoHcy and arginine- and glycine-rich substrate peptide (50). The small molecules that we identified as inhibitors of the PRMTs could interfere with either of these two adjacent but distinct regions. Using a UV cross-linking technique (43, 44), we investigate whether AMI-1 and -5 could compete for the AdoMet binding site. Purified GST-Hmt1p protein was UV-irradiated in the presence of radiolabeled AdoMet and a 50-fold molar excess of either AMI-1 or AMI-5. The cross-linking of radiolabel was monitored by separation of GST-Hmt1p by SDS-PAGE and subsequent fluorography. AMI-1 did not prevent radiolabeled AdoMet binding, whereas AMI-5 did. The structural analogue of AdoMet, sinefungin, competes for binding as expected and AdOx does not (Fig. 5). These results suggest that AMI-1 inhibits arginine methylation by inserting into the arginine-binding pocket, which can explain its high degree of PRMT specificity. On the other hand, AMI-5, although it is not an obvious AdoMet analogue, is a competitive inhibitor of AdoMet binding. AMI-5 thus

**FIG. 6. AMI-1 inhibits nuclear receptor-mediated transactivation of a luciferase reporter in MCF7 cells.** *A*, MCF7 cells were transiently transfected with an ERE-firefly reporter or an ARE-firefly reporter (+AR) and humanized CMV-*Renilla* control. After transfection, cells were treated with 10 nM estradiol to induce ERE-firefly or with 10 nM R1881 to induce ARE-firefly and the indicated amount of AMI-1. Relative activity of firefly luciferase was normalized against *Renilla* luciferase activity, and the results are expressed as normalized luciferase activity. The results are expressed as mean  $\pm$  S.D. of representative experiments done in triplicate. *Numbers in parentheses* are -fold  $IC_{50}$ . *B*, MCF7 cells were transiently transfected with an ERE-firefly reporter or an ARE-firefly reporter (+AR) with a mix of PRMT1 and PRMT4 expression plasmids (1, 10, and 100 ng). After transfection, cells were treated with 10 nM estradiol to induce ERE-firefly or with 10 nM R1881 to induce ARE-firefly in the presence of 100  $\mu$ M/well AMI-1. Luciferase activity was measured, and results are expressed as mean  $\pm$  S.D. of representative experiment done in triplicate.



likely recognizes a substructure in the AdoMet-binding fold and this may explain its ability to inhibit not only PRMT activity but also SET7 and DOT1 action (Fig. 3).

**AMI-1 Inhibits Nuclear Receptor Reporter Gene Activation—**The identification of PRMT4 (CARM1) as a GRIP1 (SRC2) binding protein led to studies showing that this methyltransferase can stimulate transcriptional activation by nuclear receptors (8). Subsequent work has demonstrated that PRMT1 also has coactivator activity, which functions synergistically with PRMT4 (25, 52). Recently, it was shown that with the engineered loss of PRMT4 function estrogen-regulated transcription is attenuated (20). We thus tested the effect that AMI-1 has on an estrogen response element (ERE) and an androgen response element (ARE) in reporter assays. MCF-7 cells were transfected with either an ERE reporter or an ARE reporter, then the cells were treated with different concentrations of AMI-1 for 2 days, and a luciferase assay was performed. Reporter activity from both nuclear receptor response elements progressively decreased as the concentration of AMI-1 was increased (Fig. 6A). At 100  $\mu$ M AMI-1 the inhibitory effect on both the ERE and ARE reporters levels off. Working at this concentration (100  $\mu$ M) of AMI-1, this inhibition can be partially overcome with the overexpression of increasing amounts of PRMT1 and PRMT4 (Fig. 6B). These results demonstrate that one of the key biological roles of PRMT1 and PRMT4, that of transcriptional coactivator, can be suppressed by AMI-1.

#### DISCUSSION

In this study we have identified nine compounds that inhibit the *in vitro* methylation of the substrate, Npl3p, by the yeast arginine methyltransferase, Hmt1p. In addition, two compounds were identified that enhance the activity of the enzyme toward the substrate. As would be expected from such a screen we did identify a compound with structural similarity to AdoMet (Fig. 1C, AMI-2) and a set of molecules that are structurally similar to each other (Fig. 1C, AMI-4, -5, and -6). Two of the compounds (Fig. 1C, AMI-1 and -9) are structurally symmetrical, and this may reflect the fact that PRMT1 and Hmt1p (39, 50) are dimers and that the active sites are symmetrical. Thus, a single symmetrical compound could inhibit both PRMT molecules within the active dimer. AMI-1, which is a symmet-

rical sulfonated urea, was identified as the lead compound from this screen for the following reasons: 1) it inhibits arginine methylation but not lysine methylation (Figs. 2 and 3); 2) it does not interfere with AdoMet-binding (Fig. 5); 3) it can inhibit endogenous cellular PRMT activity (Fig. 4, B–D); 4) it can inhibit cellular coactivator functions of PRMTs (Fig. 6); and 5) it is cell-permeable (Fig. 4, C and D). In addition, AMI-1 shares some similarity to peptidyl arginine: the urea is similar to a neutral guanidino group; the hydrophobic naphthalene ring system is a similar length to the alkyl chain of arginine, and the sulfonic acid could be aligned close to a carbonyl group of peptidyl arginine. However, there is no evidence that AMI-1 is competitive with peptidyl arginine binding or if it actually inhibits through another mechanism.

An interesting observation is that, apart from enzyme specificity, certain compounds can inhibit enzyme action on some substrates but not others. This phenomenon is observed with AMI-2, -3, and -4, which inhibit the *in vitro* enzyme action of all the PRMTs on all substrates tested (Fig. 2), except PRMT4 methylation of histone H3. Of particular interest is AMI-3, which like AMI-1 does not inhibit any of the lysine methyltransferases (Fig. 3). Similar selective specificity with regard to substrate choice is seen for the PRMT1/Hmt1p activator, AMA-2. PRMT4 methylation of PABP1 (Fig. 2) is unaffected by AMA-2, but the same compound inhibits Smb' methylation by this enzyme. AMA-2 displays similar properties on PRMT6, where it inhibits GAR but not Npl3 methylation (Fig. 2). These selective inhibitory properties of certain compounds may reflect different ways in which substrates present themselves to the enzyme active site.

AMI-1 can inhibit the *in vitro* methylation reactions performed by all five recombinantly active PRMTs (PRMT1, -3, -4, and -6 and Hmt1p). It is likely that AMI-1 binds the protein substrate-binding pocket of these enzymes, because it does not compete for the AdoMet-binding domain (Fig. 5). Structural information has demonstrated that the substrate-binding surface of Hmt1p, PRMT1, and PRMT3 are very similar thus facilitating the binding of a common compound (39, 50, 51). Although similar, the structures of the active sites are not identical (50). This raises the possibility that structural ana-

logues of AMI-1 can be generated that may be specific for individual PRMTs. Cocrystals with AMI-1 bound to PRMTs will provide valuable information on basic enzyme/inhibitor mechanism and will allow for the structural based drug design of PRMT-specific inhibitors. To increase potency of the AMIs, it may be possible to generate bisubstrate inhibitors by synthetically linking AMI-1 and an AdoMet analogue, with adjustment of the spacer arm length possibly providing varying degrees of specificity for the different PRMTs.

PRMT5 and PRMT2 were not used in this study, because they are not active as recombinant proteins (fused to GST). PRMT5, in particular, will be an important target for small molecule development because of its distinctive niche within the PRMT family (11). The unique properties of PRMT5 include: 1) its ability to generate sDMA residues (10); 2) this modification (and not aDMA) stimulates binding to Tudor domains (22); and 3) unlike PRMT1 and PRMT4, PRMT5 is reported to be involved in transcriptional repression (53).

Recently, AMI-1 was identified as a selective HIV-1 reverse transcriptase inhibitor (54). In this study structural analogues of AMI-1 were developed that had IC<sub>50</sub> values up to two orders of magnitude lower than AMI-1 itself. This suggests that superstructures of AMI-1 can be developed that will have higher sensitivity (reduced IC<sub>50</sub>) and selectivity for PRMTs. Importantly, this study also demonstrated that AMI-1 analogues are not toxic to cells, and thus, are good candidates for drug development.

Like histone acetyltransferase (HAT) activity, which is regulated by PRMTs in certain cases (17, 18), arginine methylation activity is associated with transcriptional activation. The identification of molecules that inhibit either PRMT or HAT activity may serve as lead compounds for anticancer therapy. In light of the fact that HAT and PRMT activities are synergistic in nature (with regards to transcriptional activation) (25), inhibition of either activity will likely have a profound effect on transcription. Small molecule inhibitors of HAT activity have been synthesized by design (55), and purified from natural extracts (56), and although these HAT inhibitors are effective *in vitro* they are not cell-permeable. Thus, the identification of AMI-1 as a cell-permeable PRMT inhibitor is an important step in anti-neoplastic drug development. Future refinement of this primary compound will be important, and inhibitors of PRMT4/CARM1 action are of particular interest to the medical community because of its function as a nuclear receptor coactivator.

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