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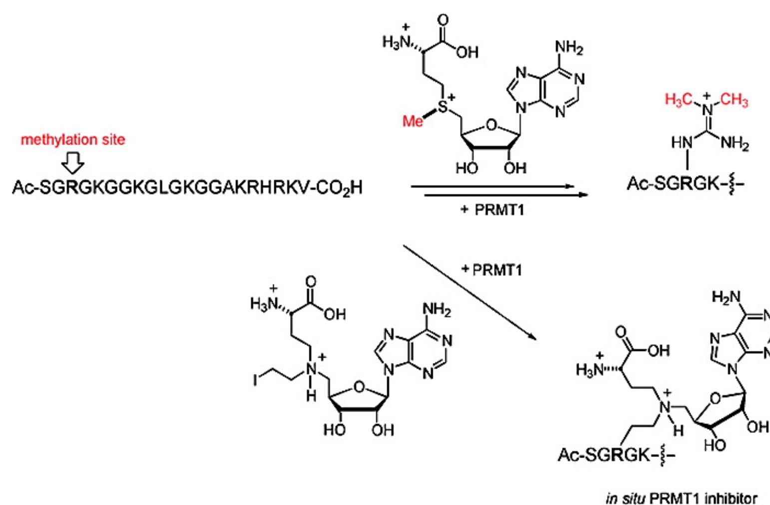

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In Situ Generation of a Bisubstrate Analogue for Protein Arginine Methyltransferase 1

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Protein arginine methyltransferases (PRMTs) catalyze the methylation of Arg residues in a variety of proteins including histones H2A, H3, and H4, as well as other proteins involved in cell signaling and RNA splicing.[1] These enzymes, which utilize (*S*)-adenosylmethionine (SAM) as the methyl donor, all catalyze the post-translational formation of monomethylated Arg residues (MMA). These residues can then be further processed to form either asymmetrically dimethylated Arg (ADMA) or symmetrically dimethylated Arg (SDMA) by Type I and Type II PRMTs, respectively (Figure 1a). In humans, at least nine PRMTs exist and are known to play physiologically important roles in numerous cellular processes, including transcriptional regulation.[2] For example, PRMT1 and PRMT4/CARM1 (coactivator associated arginine methyltransferase 1), the two best characterized PRMTs, act as transcriptional coactivators for a variety of transcription factors (e.g., estrogen receptor).[2] However, a thorough understanding of in vivo PRMT function is lacking because multiple enzymes exist and enzyme-specific inhibitors (>100-fold selectivity) have not been described. From a medical perspective, the PRMTs are also interesting because dysregulated PRMT activity appears to be associated with heart disease and cancer.[3,4] Thus PRMT-selective inhibitors may have therapeutic potential.

With this in mind, we initiated studies to characterize the molecular mechanism of PRMT catalysis[5] and develop potent and selective PRMT inhibitors. Herein we report the in situ synthesis of a bisubstrate analogue inhibitor of PRMT1 to provide proof of concept for an approach to developing PRMT selective inhibitors. While "bump and hole" and high-throughput screening (HTS) approaches have identified PRMT inhibitors,[6,7] the selectivity of these compounds is relatively poor, and inhibition constants are similar to nonspecific methyltransferase inhibitors ($K_i \geq 12\ \mu M$). Additionally, the bump and hole compounds are limited by their ability to be tested only in a system where a PRMT1 mutant can be expressed in a PRMT1$^{-/-}$ background. Similarly, the inhibitors identified by high-throughput screening are problematic because at least some inhibit structurally unrelated enzymes.[8] Thus, selective PRMT inhibitors are needed.

We initially focused on the synthesis of a PRMT1 inhibitor because this isozyme is the predominant PRMT in vivo, is dysregulated in heart disease,[4] and its regiospecificity is well-characterized.[5] The approach described here takes advantage of the inherent binding affinity of PRMT1 for both SAM and a cognate peptide substrate. Our approach also exploits the PRMT1-directed synthesis of a bisubstrate analogue from a known PRMT1 substrate and a recently described SAM congener (Figure 1b), 5′-(diaminobutyric acid)-*N*-iodoethyl-5′-deoxyadenosine ammonium hydrochloride (AAI).[9] While SAM-dependent DNA and natural product methyltransferases are known to transfer this synthetic cofactor,[9] the work described herein represents the first use of AAI by a protein methyltransferase and the first demonstration that AAI can be used to generate methyltransferase inhibitors.

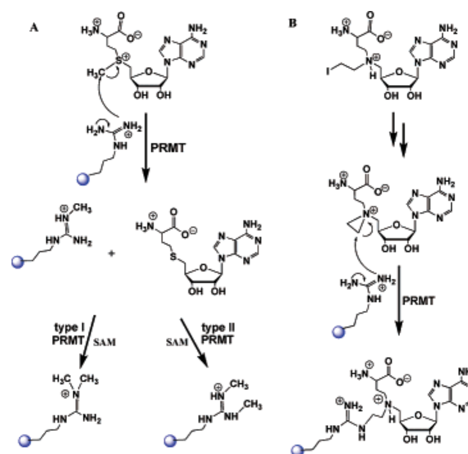

Figure 1. Reactions catalyzed by PRMTs and modification of an arginine residue with AAI. (A) Mono- and dimethylation of arginine residues by PRMTs. (B) The reasonably stable mustard forms the aziridinium ion in situ[13] which when reacted in the presence of PRMT1 produces the bisubstrate analogue.

To initially evaluate the viability of our approach for identifying PRMT-targeted bisubstrate analogues, PRMT1 was incubated with AAI, or D2AAI, a dideutero-containing analogue, and the AcH4-21 peptide. The primary structure of this peptide corresponds to the N-terminus of histone H4, a known PRMT1 substrate, and contains Arg3, a site of PRMT-1-directed methylation.[10] Significantly, this small substrate is methylated with comparable kinetics to histone H4.[5] As depicted in Figure 2, MALDI-MS experiments show respective mass shifts of 393 and 395 Da for peptides incubated with AAI and D2AAI, in agreement with the expected mass shift for a singly modified peptide. Control reactions incubated in the absence of PRMT1 did not show any modification, indicating that PRMT1 promotes the transfer of this SAM-like moiety to peptide substrates. AAI binding to the SAM binding pocket of PRMT1 is required for this activity because AAI transfer could be prevented by adding (*S*)-adenosylhomocysteine (SAH), a product inhibitor of PRMT1, or sinefungin, a SAM analogue, to the incubation mixture (Figure S1).

Additional experiments were performed to determine whether PRMT1 promotes the regiospecific transfer of (D2)AAI to Arg3. First, PRMT1 and AAI (or D2AAI) were incubated with the H4-21R3K peptide. As expected, this peptide was not modified (Figure 2, Table S1), consistent with the fact that it is an extremely poor PRMT1 substrate.[5] In contrast, PRMT1 was able to promote the transfer of the D2AAI moiety to the AcH4-15 peptide, a reasonably good PRMT1 substrate[5] whose sequence is based on the N-terminal 15 amino acids of histone H4 and contains only a single Arg residue

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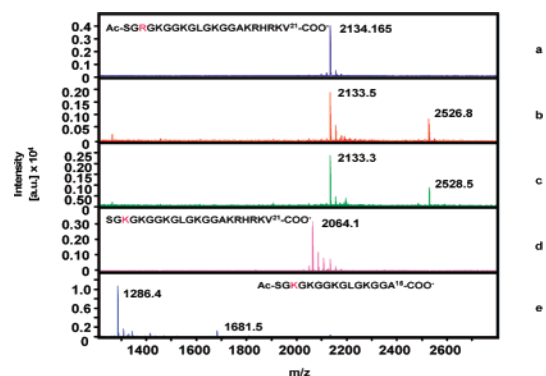

Figure 2. PRMT1-directed synthesis of a bisubstrate analogue. Mass spectra of the AcH4-21 peptide substrate (25 μM) plus AAI (250 μM) incubated in the absence (a) or presence of PRMT1 (b). Experiments with PRMT1 (5 μM) and D2AAI (250 μM) are also depicted with AcH4-21 (c), H421R3K (d), and H4-15 (e). Reactions were allowed to proceed at 37 °C for 15 min.

Table 1. Inhibitory Studies

inhibitor	IC_{50} (μM)
AAI[a,b]	18.5 ± 4.2
D2AAI[a,b]	11.9 ± 3.3
AMI-1[a,b]	350 ± 36
SAH[a,b]	8.3 ± 0.6

[a] [SAM] = 15 μM. [b] [AcH421] = 25 μM.

corresponding to Arg3. In total, these results demonstrate that PRMT1-directed AAI transfer is regiospecific.

The time and enzyme concentration dependence of D2AAI transfer was also evaluated. The results demonstrate that transfer relies on enzyme concentration but reaches near maximal levels on a short time scale, that is, extended incubations did not improve the yield of the AcH4-21−D2AAI conjugate (Figure S2). Although this may be due to the inherent instability of AAI, we also considered the possibility that the lack of time dependence is due to the generation of a bisubstrate analogue inhibitor.

To evaluate this possibility, IC_{50}'s were determined. To facilitate bisubstrate analogue formation, PRMT1, the AcH4-21 peptide, and various concentrations of D2AAI were preincubated for 10 min prior to enzyme assay. D2AAI transfer was detected in parallel. The results of these experiments demonstrate that D2AAI inhibits PRMT1 activity with similar potency to SAH[5] (Table 1, Figure S3). Similar results were obtained for AAI. While inhibition was modest under these conditions, only a small fraction (estimated to be less than 22% (Figure S4)) of the compound was transferred to the peptide. As such, the true inhibition constants are likely to be considerably lower. It is also significant that the observed IC_{50}'s are ~10-fold lower than those observed for Arg methyltransferase inhibitor 1 (AMI-1), a PRMT inhibitor identified by HTS.[7] Note that inhibition of PRMT1 by AAI is reversible, as demonstrated by dialysis experiments (Figure S5).

To evaluate selectivity, the relative inhibition of PRMT1 and PRMT4/CARM1 was compared at a single concentration of the AcH4-21−D2AAI conjugate (50 μM)—the inhibitor was generated chemoenzymatically and small quantities were purified by HPLC. These inhibition studies demonstrate that the AcH4-21−D2AAI conjugate preferentially inhibits PRMT1 by >4.4-fold (Figure S6)—PRMT1 activity is decreased to <12.5% of control versus 55% for PRMT4/CARM1.

The ability of PRMT1 to catalyze the transfer of AAI to a peptide substrate and in the process generate a bisubstrate analogue provides proof of concept for the chemoenzymatic generation of PRMT-targeted bisubstrate analogues. Although these peptide-based bisubstrate analogues are unlikely to possess drug-like properties, they will be useful for "chemical genetics" studies of PRMT function in vitro and in vivo, analogously to peptide-based histone acetyltransferase (HAT) inhibitors.[11] As described for the HAT inhibitors, the transport of these compounds into cells could be achieved by microinjection, permeabilizing reagents, or attachment to any one of a number of cell-penetrating peptides.[12] Moreover, compounds with improved bioavailability could be identified by incubating PRMT1 and AAI with a library of small molecule amine-, amidine-, or guanidinium-containing compounds. Thus, the chemoenzymatic approach described here will lead to the development of potent and selective PRMT inhibitors that will be useful for defining the roles of these enzymes under physiological and pathological conditions.

The ability of a PRMT to catalyze the transfer of AAI is also significant because appropriate AAI modifications (e.g., azide or alkyne functionalization) are likely to permit proteomic applications, including the identification of novel PRMT substrates. Additionally, such compounds could be used to identify the determinants of substrate selectivity for a particular isozyme. It should also be possible to extend this technique to generate bisubstrate analogues for other SAM-dependent methyltransferases (e.g., the lysine methyltransferases) and thereby provide potent and selective inhibitors for a wide range of enzymes.

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Supporting Information Available: Tables S1 and S2, Figures S1−S5, materials, and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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