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Discovery of Bisubstrate Inhibitors of Nicotinamide *N*-Methyltransferase (NNMT)

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Abstract

Nicotinamide *N*-methyltransferase (NNMT) catalyzes the *N*-methylation of pyridine-containing compounds using the cofactor *S*-5'-adenosyl-L-methionine (SAM) as the methyl group donor. Through the regulation of the levels of its substrates, cofactor, and products, NNMT plays an important role in physiology and pathophysiology. Overexpression of NNMT has been implicated in various human diseases. Potent and selective small-molecule NNMT inhibitors are valuable chemical tools for testing biological and therapeutic hypotheses. However, very few NNMT

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Accession Codes

The structure of human NNMT in complex with **6** has been deposited under PDB ID 6B1A. Authors will release the atomic coordinates and experimental data upon article publication.

Notes

The authors declare the following competing financial interest(s): J.F. is an employee and shareholder of Accutar Biotechnology. J.J. is a consultant of Accutar Biotechnology.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmed-chem.7b01422.

¹H and ¹³C NMR spectra of compound **6**; Docking analysis of **7** in the binding sites of hNNMT; Docking analysis of **6** in the binding sites of DOT1L and SMYD2; Kinetic parameter determination for NNMT; Binding affinity of SAH to hNNMT and binding affinity of nicotinamide to hNNMT in the presence of SAH; Overlay of the crystal structure of the hNNMT (PDB 6B1A) with the crystal structure of mouse NNMT (PDB 5XVK); Proteins in the methyltransferase selectivity panel; Crystallography data and refinement statistics (PDF)

Molecular formula strings (CSV)

Docking process movie for **6** (MPG)

inhibitors have been reported. Here, we describe the discovery of a bisubstrate NNMT inhibitor MS2734 (**6**) and characterization of this inhibitor in biochemical, biophysical, kinetic, and structural studies. Importantly, we obtained the first crystal structure of human NNMT in complex with a small-molecule inhibitor. The structure of the NNMT–**6** complex has unambiguously demonstrated that **6** occupied both substrate and cofactor binding sites. The findings paved the way for developing more potent and selective NNMT inhibitors in the future.

Graphical Abstract

INTRODUCTION

Nicotinamide *N*-methyltransferase (NNMT) catalyzes the transfer of the methyl group from the cofactor *S*-5'-adenosyl-L-methionine (SAM) to the N1-positions of nicotinamide and a variety of structurally related pyridine-containing compounds, such as thionicotinamide, quinoline, isoquinoline, 3-acetylpyridine, and others.^{1,2} The NNMT gene is predominantly expressed in liver, and significant expression levels of NNMT are detected in adipose tissue, kidney, lung, skeletal muscle, placenta, and heart.^{3,4} The function of NNMT has traditionally been assigned to the nicotinamide clearance and xenobiotic detoxification.^{1,5} Recently, more NNMT functions have been revealed in both health and diseases through modulation of the levels of its products, substrates, and cofactor.⁵ Several beneficial effects of NNMT activity have been reported through the generation of the endogenous product N1-methyl nicotinamide (**1**). For example, **1** up-regulates the reactive oxygen species (ROS) and leads to lifespan extension in *Caenorhabditis elegans*.⁶ **1** also increases the secretion of prostacyclin from endothelial cells to regulate thrombotic and inflammatory processes.⁷ In addition, **1** enhances the release of nitric oxide from endothelial cells and leads to vasodilation of blood vessels.⁸ Moreover, **1** stabilizes Sirt1 protein in liver, decreases hepatocyte fatty acid and cholesterol synthesis, and suppresses liver triglyceride, cholesterol content, and liver inflammation.⁹ However, other NNMT metabolized *N*-methylated pyridinium products, such as *N*-methyl-4-phenyl-pyridinium ion, are well-established dopaminergic toxins.¹⁰ These pyridinium metabolites have been deemed as the causative agents for Parkinson's disease.^{10,11} Indeed, NNMT expression and activity are elevated in the brain tissues of Parkinson's disease patients.^{10,11} To date, the best-studied NNMT substrate is nicotinamide, which is a biosynthetic precursor of nicotinamide adenine dinucleotide (NAD⁺), an important molecule involved in redox reactions and posttranslational modifications. The cofactor of NNMT, SAM, provides propylamine groups for polyamine biosynthesis. SAM is also the common methyl group donor for a variety of methyltransferases. By direct alteration of NAD⁺ and SAM levels, NNMT regulates the energy metabolism and functions as a regulator of adiposity.¹² NNMT expression has been

observed in white adipose tissue (WAT) of obese and diabetic mice. Knockdown NNMT with antisense oligonucleotides (ASOs) increases energy expenditure and prevents weight gain in high-fat diet mice.¹² Down-regulations of the SAM level and consequently the methylation states of histones and nonhistone proteins have been demonstrated as the pathological mechanism of NNMT overexpression in cancers.¹³ NNMT activity is elevated in a diverse set of cancers, including brain,¹⁴ thyroid,¹⁵ lung,¹⁶ liver,¹⁷ kidney,^{18,19} stomach,^{20,21} bladder,²² pancreas,²³ oral,^{24,25} and colon.²⁶ NNMT knockdown decreases cell proliferation, migration, and/or metastasis of bladder,²² kidney,¹⁹ pancreas,²³ and oral²⁵ cancer cells. Although the endogenous roles of NNMT in physiology and disease seem to be contradicting and need to be further investigated, NNMT could be a potential therapeutic target in diseases with abnormally high expression of NNMT.

Potent and selective small-molecule inhibitors of NNMT are valuable chemical tools for deciphering the complex regulatory mechanisms mediated by NNMT and for testing biological and therapeutic hypotheses associated with NNMT. However, only very few NNMT inhibitors have been reported to date (Figure 1).^{3,27–29} **1**,³ the product of the NNMT enzymatic reaction, has been widely used as an NNMT inhibitor to study the biological function of NNMT.¹² Since last year, three types of new NNMT inhibitors have been published. An NNMT covalent inhibitor, RS004 (**2**),²⁷ was reported targeting the Cys165 residue within the SAM-binding pocket, and N-methylated quinolinium compounds **3** and **4**²⁸ were disclosed as substrate mimetic inhibitors. During the preparation of this manuscript, a bisubstrate NNMT inhibitor, **5**,²⁹ was reported targeting both substrate and cofactor binding sites. However, **5** displayed modest potency ($IC_{50} = 29 \mu M$) in a biochemical assay and was not characterized in biophysical assays and selectivity assays. In fact, except for the covalent inhibitor **2**, no selectivity profiling data were provided for the reported NNMT inhibitors. In addition, no co-crystal structures of these NNMT inhibitors in complex with human NNMT (hNNMT) have been reported.

A bisubstrate inhibitor consists of two covalently linked fragments, each targeting either the substrate binding site or the cofactor binding site, thus potentially mimicking the ternary transition state of a bireactant enzymatic reaction. This inhibitor design strategy has the potential to achieve high potency and selectivity. A number of bisubstrate inhibitors have been successfully developed for a variety of enzymes, including kinases³⁰ and acetyltransferases.³¹ Inspired by the recently published ternary crystal structure of hNNMT in complex with the substrate nicotinamide and the cofactor product *S*-5'-adenosyl-L-homocysteine (SAH),³² we designed and synthesized NNMT bisubstrate inhibitors, MS2734 (**6**) and MS2756 (**7**). We characterized these compounds in a battery of biochemical and biophysical assays. In addition, using the bisubstrate inhibitor **6**, we studied the kinetic mechanism of NNMT inhibition. Furthermore, we obtained a co-crystal structure of **6** in complex with hNNMT. Here, we report the design, synthesis, and biochemical and biophysical characterization of these bisubstrate inhibitors.

RESULTS AND DISCUSSION

Design

The crystal structure of the hNNMT–nicotinamide–SAH ternary complex (PDB 3ROD) revealed that nicotinamide and SAH occupy two adjacent binding sites.³² The distance between the nicotinamide nitrogen atom and the SAH sulfur atom ranges from 3.5 to 4.2 Å.³² We thus hypothesized that by covalently linking the nicotinamide mimic group **8** and the SAM mimic moieties **9** using a 2-carbon-atom linker (total linear distance ~4.5 Å), we could generate compounds **6** and **7** as bisubstrate inhibitors of NNMT (Figure 2). We docked compound **6** into the SAH binding site of the crystal structure of the hNNMT–SAH binary complex (PDB 2IIP) by utilizing a side chain flexible docking strategy³³ to probe whether the 3-amido-phenyl moiety of **6** could bind to the substrate-binding pocket of hNNMT (Figure 3A and the docking process movie is available in the SI). Comparing our docking model with the hNNMT–nicotinamide–SAH ternary structure, we found that the 3-amido-phenyl moiety of **6** does occupy the nicotinamide binding site (Figure 3B). Moreover, the orientation of docked 3-amido-phenyl group is almost identical to that of nicotinamide in one of the four ternary structures (chain B) in the published asymmetric unit (PDB 3ROD).³² The oxygen atom of the 3-amido group interacts with Ser201 and Ser213 residues through hydrogen bonds (H-bonds), and the nitrogen atom of the 3-amido group forms a H-bond with Ser213 (Figure 3A). We also conducted similar docking studies for compound **7** (Supporting Information, Figure S1), which suggested that compound **7** can also fit into the two adjacent binding sites, but is less favorable compared with compound **6**.

Synthesis

Synthesis of compounds **6** and **7** started with the preparation of intermediate **12** from the reductive amination of commercially available starting materials **10** and 3-(2-oxoethyl)-benzonitrile **11** (Scheme 1).³⁴ Coupling of intermediate **12** with aldehydes **13**^{35,36} via another reductive amination reaction provided the nitrile intermediates **14**. After hydrolysis of the cyano group to the amido group under basic condition in the presence of hydrogen peroxide, the protecting groups on the resulting intermediates **15** were removed to provide the final products **6** and **7**.

Biochemical and Biophysical Characterization

To evaluate the biological activities of compounds **6** and **7**, we used a SAH hydrolase (SAHH)-coupled fluorescent assay.^{37,38} SAHH hydrolyzes SAH, the NNMT cofactor product, to adenosine and homocysteine. The free thiol group of homocysteine conjugates to a thiol-sensitive fluorophore (ThioGlo) and generates fluorescence signal. Thus, the methylation reaction can be followed by measuring the increase of the fluorescence intensity. We performed full kinetic characterization of NNMT using SAHH-coupled assay and determined K_m values for nicotinamide ($20 \pm 3 \mu\text{M}$) and SAM ($24 \pm 6.8 \mu\text{M}$) with a k_{cat} of $4.1 \pm 0.2 \text{ min}^{-1}$ (Supporting Information, Figure S3). The K_m value we obtained for nicotinamide was lower than what has been reported in the literature using end-point assays.^{2,3,28,29,32} The differences are likely due to using different assay conditions such as temperature, pH, and additives.

Under the SAHH-coupled assay conditions, both compounds **6** and **7** inhibited the methyltransferase activity of NNMT with IC₅₀ values of $14 \pm 1.5 \mu\text{M}$ (Hill Slope: 1.1) and $160 \pm 1 \mu\text{M}$, respectively (Figure 4A). Next, we confirmed the binding of compounds **6** and **7** to hNNMT using isothermal titration calorimetry (ITC). Consistent with the SAR trend from the biochemical assay, compound **6** showed better binding affinity ($K_d = 2.7 \pm 0.2 \mu\text{M}$) than compound **7** ($K_d = 42.8 \pm 6.3 \mu\text{M}$) (Figure 4B). These assay results indicate that the length of the amino acid moiety of the bisubstrate inhibitors is important for NNMT potency and binding affinity. These assay results support the prediction from our docking studies that compound **7** is less favorable to bind the NNMT substrate and cofactor binding site compared with compound **6**.

Selectivity Assessment

To assess selectivity of compound **6**, we tested it against 34 histone, DNA and RNA methyltransferases, and a histone acetyltransferase (Supporting Information, Table S1). We were pleased to find that compound **6** did not inhibit the majority of these enzymes at up to $50 \mu\text{M}$ (Figure 4C). Out of these 35 examined enzymes, only four enzymes (DOT1L, PRMT7, BCDIN3D, and SMYD2) were significantly inhibited with IC₅₀ and Hill slope (HS) values of $1.3 \pm 0.2 \mu\text{M}$ (HS: 1.1), $20 \pm 2 \mu\text{M}$ (HS: 1.1), $40 \pm 2 \mu\text{M}$ (HS: 1.3), and $62 \pm 7 \mu\text{M}$ (HS: 0.6), respectively (Figure 4D). The selectivity profiling of **6** over other methyltransferases in general indicates that the bisubstrate inhibitor design strategy could provide potent and selective NNMT inhibitors. Interestingly, **6** inhibited DOT1L with low μM potency. This result is consistent with previous reports that SAM analogs are effective DOT1L inhibitors.³⁴ In addition, **6** could serve as a potential starting point for developing selective inhibitors of PRMT7 and BCDIN3D as no selective inhibitors of PRMT7 and BCDIN3D have been reported to date.

To gain insights into the structural basis of **6** binding to these off-targets, we docked compound **6** into two previously published crystal structures: DOT1L (PDB: 3QOW)³⁹ and SMYD2 (PDB: 3TG4)⁴⁰ (Supporting Information, Figure S2). Our docking models suggest that the amidophenyl group of **6** occupies the substrate binding sites of both DOT1L and SMYD2, which is consistent with our selectivity assay results. It appears that, although the amino acid residues and the size and shape of the substrate binding sites of NNMT, DOT1L, and SMYD2 are different, these substrate binding sites can accommodate a small moiety such as the amidophenyl group of inhibitor **6**. We believe it is possible to achieve selectivity among these targets by modifying the amidophenyl group.

Mechanism of Action (MOA) Studies

Bisubstrate inhibitors are useful tools for studying enzymatic mechanisms.³¹ Specifically, kinetic studies of bisubstrate inhibitors could provide valuable information to determine whether the enzymes catalyze the biological reaction through a random or an ordered mechanism.³¹ To assess the MOA of **6**, we evaluated the effect of SAM and nicotinamide concentrations on the rate of the enzymatic reaction at various concentrations of the bisubstrate inhibitor **6**. As illustrated in Figure 5A, the bisubstrate inhibitor **6** is SAM competitive as evidenced by changes in K_m values for SAM but no change in V_{max} values at various concentrations of the inhibitor (the $1/\text{rate}$ versus $1/[\text{SAM}]$ intersecting on the y -axis

of the Lineweaver–Burk plot). As illustrated in Figures 5B, **6** is noncompetitive with the nicotinamide substrate, with no change in K_m values for nicotinamide but significant changes in V_{max} values as the concentration of **6** was increased (1/rate versus 1/[nicotinamide] intersecting on the x -axis of the Lineweaver–Burk plot). Although both SAM ($K_d = 56.4 \pm 9.5 \mu\text{M}$) and compound **6** ($K_d = 2.7 \pm 0.2 \mu\text{M}$) bound to hNNMT in ITC experiments (Figures 4B and 5C), reliably measurable nicotinamide binding to hNNMT was not observed in the absence of the cofactor (Figure 5D). In the presence of SAH, however, nicotinamide was able to bind to hNNMT (Supporting Information, Figure S4). These binding phenomena suggest a possible ordered mechanism for hNNMT enzymatic activity, where SAM binds first, followed by nicotinamide binding. This is consistent with previously reported competitive patterns that indicate an ordered enzymatic kinetic mechanism.^{31,41} However, recently a random mechanism has been reported for NNMT activity,⁴² where SAM and quinoline would independently bind to NNMT. The authors also observed that both SAM and quinoline bound to the complementary binary complexes with a much higher affinity (20-fold) compared to binding to the apoenzyme.⁴² This may be consistent with the nicotinamide binding we observed in the absence and presence of SAH (Figure 5D and Supporting Information, Figure S4).

Co-crystal Structure of Compound **6** in Complex with hNNMT

To elucidate the interactions between hNNMT and compound **6** at an atomic level, we obtained an X-ray co-crystal structure of hNNMT in complex with **6** at 2.3 Å (PDB 6B1A, Figure 6A,B, and Supporting Information, Table S2). The crystal asymmetric unit contains four independent, conformationally similar NNMT molecules. Overlay of these molecules gives rmsd values ranging from 0.105 to 0.205 Å. Electron density of compound **6** is unambiguously defined in all of the NNMT molecules. We superimposed our hNNMT–**6** structure with the published hNNMT–nicotinamide–SAH complex (Figure 6C).³² Consistent with our bisubstrate inhibitor design hypothesis and docking model study, compound **6** does occupy both cofactor and substrate binding sites. The cofactor mimicking moiety of compound **6** overlays nicely with SAH, and the majority of the ligand-protein interactions in this binding pocket are conserved. For example, the carboxylate oxygen atoms form H-bonds with four hydroxyl groups from the side chains of Tyr20, Tyr25, Tyr69, and Thr163. In addition, the ribose hydroxyl groups interact with the side chains of Asp85 and Asn90 through H-bonds. Moreover, the adenine moiety is sandwiched between Tyr86 and Ala169, and the adenine N1 nitrogen forms a H-bond with the main chain amide nitrogen of Val143. Compound **6** also possesses a few different interactions compared with SAH in the cofactor binding site. While the homocysteine amino group of SAH forms H-bonds with Gly63, Thr163, and a water molecule, the corresponding amino group of **6** orients to a different direction and locates within the hydrogen bond distance of two additional residues, Ser64 and Thr67. Other different interactions occur at the adenine N6-amino group. Besides the conserved H-bond with the Asp142, compound **6** indirectly interacts with Ala168 through water mediated H-bonds. The 3-amido phenyl group of compound **6** nests nicely between Leu164 and Tyr204 at the nicotinamide-binding site. Three important H-bond interactions with Ser201 and Ser203 are conserved. Compared to nicotinamide, the 3-amido phenyl moiety projects a little bit deeper in the substrate-binding site. Therefore, the direct hydrogen bond between the amide nitrogen of nicotinamide and Asp197 is lost. Instead,

water-molecule-mediated H-bonds are observed between the amide nitrogen of compound **6** and three residues (Ala168, Leu164, and Asp197). Since the N1-nitrogen of nicotinamide was removed on **6**, the interaction between the nicotinamide N1-nitrogen and Tyr20 is lost.

During the preparation of this manuscript, crystal structures of monkey and mouse NNMT in complex with **1** and SAH were published.⁴³ Our hNNMT-**6** structure overlays very well with these two new structures with almost identical ligand-protein interactions (Figure 6D and Supporting Information Figure S5). Interestingly, while the 3-amido phenyl group of compound **6** is located deeper in the substrate binding site compared to nicotinamide, this group sits at almost the same location as **1** in the monkey NNMT-**1** complex (Figure 6C,D).

CONCLUSIONS

Using a structure-based bisubstrate inhibitor design strategy, we designed and synthesized compounds **6** and **7**. In a biochemical assay, compound **6** inhibited the hNNMT methyltransferase activity with an IC_{50} of $14 \pm 1.5 \mu M$. We confirmed that compound **6** was a tight binding ligand for hNNMT using ITC ($K_d = 2.7 \pm 0.2 \mu M$). Compound **7** was less potent than compound **6** in biochemical and biophysical assays, consistent with our modeling results. Compound **6** was selective for hNNMT over a broad range of methyltransferases with the exception of DOT1L and PRMT7. In MOA studies, compound **6** was competitive with the cofactor SAM and noncompetitive with the substrate nicotinamide. We obtained the crystal structure of human NNMT in complex with compound **6**, which has clearly demonstrated that **6** occupied both substrate and cofactor binding sites, thus validating our bisubstrate inhibitor design strategy. These results are valuable for the research community to develop the next generation of NNMT inhibitors with improved potency and selectivity.

EXPERIMENTAL SECTION

Chemistry General Procedures

HPLC spectra for all compounds were acquired using an Agilent 1200 Series system with DAD detector. Chromatography was performed on a 2.1×150 mm Zorbax 300SB- C_{18} $5 \mu m$ column with water containing 0.1% formic acid as solvent A and acetonitrile containing 0.1% formic acid as solvent B at a flow rate of 0.4 mL/min. The gradient program was as follows: 1% B (0–1 min), 1–99% B (1–4 min), and 99% B (4–8 min). High-resolution mass spectra (HRMS) data were acquired in positive-ion mode using an Agilent G1969A API-TOF with an electrospray ionization (ESI) source. Nuclear Magnetic Resonance (NMR) spectra were acquired on a Bruker DRX-600 spectrometer (600 MHz 1H , 150 MHz ^{13}C). Chemical shifts are reported in ppm (δ). Preparative HPLC was performed on Agilent Prep 1200 series with UV detector set to 254 nm. Samples were injected into a Phenomenex Luna 250 $\times$ 30 mm, $5 \mu m$, C_{18} column at room temperature. The flow rate was 40 mL/min. A linear gradient was used with 10% (or 50%) of MeOH (A) in H_2O (with 0.1% TFA) (B) to 100% of MeOH (A). HPLC was used to establish the purity of target compounds. All final compounds had >95% purity using the HPLC methods described above.

3-(2-(((3aR,4R,6R,6aR)-6-(6-Amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl)amino)ethyl)-benzonitrile (12)—To a solution of 3-(2-oxoethyl)benzonitrile **11** (0.15 g, 1 mmol) in 25 mL DCM was added **12** (0.46 g, 1.5 mmol) followed by 5 drops of AcOH. The resulting mixture was stirred for 30 min before NaBH(OAc)₃ (0.32 g, 1.5 mmol) was added. After being stirred for 16 h at rt, the reaction was quenched with saturated NaHCO₃ and extracted with EtOAc (5×). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated. The residue was purified with silica gel column to provide desired product **12** (0.31 g, 71%). ¹H NMR (600 MHz, CD₃OD) δ 8.49 (s, 1H), 8.44 (s, 1H), 7.61–7.58 (m, 2H), 7.54 (d, *J* = 8.0 Hz, 1H), 7.48 (t, *J* = 7.9 Hz, 1H), 6.39 (d, *J* = 2.1 Hz, 1H), 5.41 (dd, *J* = 6.3, 2.2 Hz, 1H), 5.16 (dd, *J* = 6.3, 4.0 Hz, 1H), 4.59 (dt, *J* = 10.0, 3.6 Hz, 1H), 3.61–3.50 (m, 2H), 3.27 (t, *J* = 8.2 Hz, 2H), 3.02–2.98 (m, 2H), 1.61 (s, 3H), 1.38 (s, 3H). HRMS calcd for C₂₂H₂₅N₇O₃ + H: 436.2097; found: 436.2094 [M + H]⁺.

tert-Butyl (S)-5-(((3aR,4R,6R,6aR)-6-(6-Amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl)(3-cyanophenethyl)amino)-2-((bis(tert-butoxycarbonyl)amino)-pentanoate (14a, Compound 14 when n = 2)—To a solution of **12** (0.065 g, 0.15 mmol) in 5 mL DCM was added aldehyde **13a** (compound **13** when *n* = 2) (0.058 g, 0.15 mmol) followed by 2 drops of AcOH. The resulting mixture was stirred 30 min before NaBH(OAc)₃ (0.048 g, 0.23 mmol) was added. After being stirred for 16 h at rt, the reaction was quenched with saturated NaHCO₃ and extracted with EtOAc (5×). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated. The residue was purified with silica gel column to provide desired product **14a** (0.074 g, 61%). ¹H NMR (600 MHz, CDCl₃) δ 8.28 (s, 1H), 7.86 (s, 1H), 7.39 (dt, *J* = 6.8, 1.9 Hz, 1H), 7.33–7.31 (bs, 1H), 7.26–7.24 (m, 2H), 5.98 (d, *J* = 2.2 Hz, 1H), 5.91 (s, 2H), 5.45 (dd, *J* = 6.5, 2.2 Hz, 1H), 4.88 (dd, *J* = 6.4, 3.3 Hz, 1H), 4.63 (dd, *J* = 9.6, 5.3 Hz, 1H), 4.25 (td, *J* = 6.7, 3.3 Hz, 1H), 2.75–2.68 (m, 1H), 2.65–2.54 (m, 5H), 2.51–2.42 (m, 2H), 1.98–1.90 (m, 1H), 1.80–1.69 (m, 1H), 1.54 (s, 3H), 1.43 (s, 18H), 1.38 (s, 11H), 1.33 (s, 3H). HRMS calcd for C₄₁H₅₈N₈O₉ + H: 807.4405; found: 807.4402 [M + H]⁺.

tert-Butyl (S)-4-(((3aR,4R,6R,6aR)-6-(6-Amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl)(3-cyanophenethyl)amino)-2-((bis(tert-butoxycarbonyl)amino)-butanoate (14b, Compound 14 when n = 1)—Intermediate **14b** (66%) was prepared according to the procedure for **14a** and further purified by reverse-phase HPLC. ¹H NMR (600 MHz, CD₃OD) δ 8.46 (s, 1H), 8.44 (s, 1H), 7.62 (d, *J* = 6.8 Hz, 1H), 7.58 (s, 1H), 7.54–7.44 (m, 2H), 6.41 (d, *J* = 2.1 Hz, 1H), 5.37 (dd, *J* = 6.4, 2.1 Hz, 1H), 5.17 (dd, *J* = 6.3, 4.3 Hz, 1H), 4.77–4.67 (m, 2H), 3.91 (dd, *J* = 14.1, 10.4 Hz, 1H), 3.80 (dd, *J* = 14.0, 2.3 Hz, 1H), 3.53–3.40 (m, 3H), 3.30–3.23 (m, 1H), 3.14–2.91 (m, 2H), 2.53–2.41 (m, 1H), 2.06–1.93 (m, 1H), 1.65 (s, 3H), 1.49 (s, 18H), 1.44 (s, 9H), 1.39 (s, 3H). HRMS calcd for C₄₀H₅₆N₈O₉ + H: 793.4249; found: 793.4251 [M + H]⁺.

tert-Butyl (S)-5-(((3aR,4R,6R,6aR)-6-(6-Amino-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl)(3-

carbamoylphenethyl)amino)-2-((bis(tert-butoxycarbonyl))amino)-pentanoate (15a, Compound 15 when n = 2)—To a solution of **14a** (0.040 g, 0.05 mmol) in DMSO (1 mL) was added K₂CO₃ (0.028 g, 0.2 mmol). The mixture was cooled to 0 °C and treated with H₂O₂ (0.1 mL). The reaction mixture was warmed to rt and stirred for 3 h at rt. The reaction was diluted with water and extracted with EtOAc (3×). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated. The residue was purified by silica gel column to provided **15a** (0.034 g, 82%). ¹H NMR (600 MHz, CDCl₃) δ 8.28 (s, 1H), 7.99 (s, 1H), 7.64 (d, *J* = 7.7 Hz, 1H), 7.48 (s, 1H), 7.23 (t, *J* = 7.6 Hz, 1H), 7.13 (d, *J* = 7.6 Hz, 1H), 7.02 (s, 1H), 6.44 (s, 1H), 6.42 (s, 2H), 6.06 (d, *J* = 2.0 Hz, 1H), 5.45 (d, *J* = 5.8 Hz, 1H), 4.97 (s, 1H), 4.65 (dd, *J* = 9.6, 5.2 Hz, 1H), 4.43 (s, 1H), 2.98–2.85 (m, 2H), 2.84–2.73 (m, 2H), 2.72–2.61 (m, 4H), 2.05–1.96 (m, 1H), 1.88–1.77 (m, 1H), 1.57 (s, 3H), 1.45 (s, 18H), 1.41 (s, 11H), 1.34 (s, 3H). MS (ESI) *m/z* 825.6 [M + H]⁺

(S)-2-Amino-5-(((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)(3-carbamoylphenethyl)-amino)pentanoic acid (6)—The intermediate **15a** (0.034 g, 0.041 mmol) was dissolved in dioxane (0.25 mL). The resulting solution was cooled to 0 °C and treated with HCl (4 M in dioxane, 0.25 mL). The reaction mixture was warmed to rt and stirred for 4 h before 2 drops of water was added. The resulting mixture was stirred for another 4 h at rt. After concentration, the residue was dissolved in MeOH (0.1 mL). To the solution Et₂O was added to triturate the desired product **6** as a white solid. After filtration and wash with Et₂O, the desired product **6** (0.015 g, 69%) was obtained. ¹H NMR (600 MHz, CD₃OD) δ 8.57 (s, 1H), 8.46 (s, 1H), 7.82–7.65 (m, 2H), 7.44–7.29 (m, 2H), 6.18 (s, 1H), 4.74 (d, *J* = 28.4 Hz, 1H), 4.55 (t, *J* = 8.0 Hz, 1H), 4.49 (t, *J* = 5.5 Hz, 1H), 4.07 (s, 1H), 3.94–3.82 (m, 1H), 3.81–3.72 (m, 1H), 3.60–3.49 (m, 2H), 3.49–3.38 (m, 2H), 3.20–3.04 (m, 2H), 2.14–2.00 (m, 3H), 2.00–1.89 (m, 1H). ¹³C NMR (151 MHz, CD₃OD) δ 171.8, 171.2, 152.0, 149.7, 145.8 (broad peak), 144.9 (broad peak), 137.9, 135.6, 133.4, 130.1, 129.2, 127.4, 121.1, 92.0, 80.4 (0.5C), 79.7 (0.5C), 74.6, 73.5, 56.8 (0.5C), 56.0 (0.5C), 55.7 (0.5C), 55.3 (0.5C), 54.2, 53.2, 30.8 (0.5C), 30.2 (0.5C), 28.5, 21.2. Note: A couple of peaks are broad and overlap: 145.8 and 144.9. Four sets of peaks are doubled peaks: 80.4 and 79.7; 56.8 and 56.0; 55.7 and 55.3; 30.8 and 30.2. Similar doubled peaks have been observed in other SAM analogs.⁴⁴ HRMS calcd for C₂₄H₃₂N₈O₆ + H: 529.2523; found: 529.2524 [M + H]⁺.

(S)-2-Amino-4-(((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl)(3-carbamoylphenethyl)-amino)butanoic acid (7)—Compound **7** (46% over 2 steps) was prepared according to the procedure for **6** and purified by reverse-phase HPLC. ¹H NMR (600 MHz, CD₃OD) δ 8.40 (s, 1H), 8.32 (s, 1H), 7.76–7.65 (m, 2H), 7.38–7.25 (m, 2H), 6.11 (d, *J* = 4.1 Hz, 1H), 4.75 (t, *J* = 4.7 Hz, 1H), 4.54–4.48 (m, 1H), 4.46 (t, *J* = 5.4 Hz, 1H), 4.00–3.93 (m, 1H), 3.82–3.73 (m, 1H), 3.72–3.65 (m, 1H), 3.63–3.57 (m, 1H), 3.55–3.42 (m, 3H), 3.15–3.02 (m, 2H), 2.46–2.34 (m, 1H), 2.22–2.13 (m, 1H). HRMS calcd for C₂₃H₃₀N₈O₆ + H: 515.2367; found: 515.2365 [M + H]⁺.

Side Chain Flexible Docking Study of NNMT with Inhibitors 6 and 7

Multiple 3D structures of compounds **6** and **7** were created from smile files. The hNNMT structure (PDB 2IIP) was used as the template for initial docking of the ligands by deep neural network-based docking program (Orbital).³³ As illustrated in the movie in the Supporting Information, upon obtaining initial docking poses of the ligands, protein side chains of 2IIP were allowed to cofold in the presence of the top-scoring dock ligand by side chain prediction platform.³³ After multiple rounds of the docking–refolding process, the converged protein–ligand complex structure was created as the final docking result.

hNNMT Protein Expression and Purification for Biochemical Assay and MOA Studies

DNA fragment encoding hNNMT (residues 1–264) was amplified by PCR and subcloned into the pET28a-LIC vector, downstream of the poly histidine coding region. The overexpression of the recombinant proteins in *E. coli* BL21 (DE3) pRARE2-V2R was induced by addition of 0.5 mM isopropyl-1-thio-D-galactopyranoside (IPTG) and overnight incubation at 16 °C. Harvested cells were resuspended in 20 mM Tris-HCl buffer, pH 7.5, containing 500 mM NaCl, 5 mM imidazole and 5% glycerol, 1 mM PMSF, 1 mM DTT, and Roche complete EDTA-free protease inhibitor cocktail tablet. The cells were lysed chemically by rotating 30 min with CHAPS (final concentration of 0.5%) and 2.5 μ g benzonase nuclease, followed by sonication for 5 min (Sonicator 3000, Misoni). The crude extract was clarified by high-speed centrifugation (60 min at 36,000 $\times g$ at 4 °C). After centrifugation, the lysate was then loaded onto a Ni-NTA resin (Qiagen) column. The column was washed and eluted by running 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5% glycerol, containing 30 mM and 250 mM imidazole, respectively. The purity of the protein was judged by SDS-PAGE gel, and the pure protein solution was dialyzed overnight against 20 mM Tris, pH 7.5, 250 mM NaCl, 1 mM DTT, and 5% glycerol and then concentrated, aliquoted, flash frozen in liquid nitrogen and stored at –80 °C.

hNNMT Biochemical Assays and Enzyme Kinetics Study

SAHH-coupled assay was used as previously described³⁸ to determine the NNMT kinetic parameters, the IC₅₀ values of **6** and **7**, and the mechanism of action (MOA) of compound **6**. 200 nM hNNMT was used in all reactions with nicotinamide (cat. no. N-3376, Sigma) and SAM (cat. no. S018, AK scientific) as substrates in the presence of 5 μ M SAHH (purified in house) and 15 μ M ThioGlo (cat. no. 595501; Calbiochem, Gibbstown, NJ). The methylation assays were performed in 50 mM Tris, pH 7.5, 0.01% Triton X-100 with a constant DMSO concentration of 2% (v/v). IC₅₀ values for compounds **6** and **7** were determined in triplicate by varying the concentration of inhibitor from 250 nM to 250 μ M with SAM and nicotinamide concentrations kept constant at 25 μ M ($\sim K_m$ value of each substrate). Data were then fit to Four Parameter Logistic equation using GraphPad Prism software 7.02. To study the MOA of compound **6**, 200 μ M fixed concentration of nicotinamide and varying concentrations of SAM (from 12.5 to 200 μ M) or 250 μ M fixed concentration of SAM and varying concentrations of nicotinamide (from 12.5 to 200 μ M) were included in reactions at 0, 1, 2, 4, 8, 16, 31, 63, 125, and 250 μ M of compound **6**. Reactions (in quadruplicates) were initiated by adding SAM and followed by measuring the increase in fluorescence using Synergy 4 microplate reader with 360/40 nm excitation filter and 528/20 nm emission filter

in 384-well plate format. The data were analyzed by enzyme kinetic module of SigmaPlot software 11.0. The relationships between the measured activity and the concentration of SAM or nicotinamide for various fixed compound **6** concentrations were fitted globally with equations describing competitive and noncompetitive inhibition with respect to SAM and nicotinamide, respectively.

Selectivity Assays

Effect of compound **6** on methyltransferase activity of G9a, GLP, SUV39H1, SUV39H2, SETDB1, SETD8, SUV420H1, SUV420H2, SETD7, MLL1 pentameric complex, MLL3 pentameric complex, EZH2 trimeric complex, PRMT1, PRMT3, PRMT4, PRMT5/MEP50 complex, PRMT6, PRMT7, PRMT8, PRMT9, PRDM9, SETD2, SMYD2, SMYD3, DNMT1, BCDIN3D, METTL3_METTL14 complex, and acetyltransferase activity of EP300 were assessed by monitoring the incorporation of tritium-labeled methyl/acetyl group to substrates using Scintillation Proximity Assay (SPA) as previously described.^{45,46} Assays were performed in a 10 μ L reaction mixture containing ³H-SAM or ³H-acetyl coenzyme A for EP300 (PerkinElmer; www.perkinelmer.com) at substrate concentrations close to K_m values for each enzyme. Two concentrations (10 μ M and 50 μ M) for all selectivity assays and titrations from 200 nM to 200 μ M of compound **6** for hit IC₅₀ determinations were used. To stop the enzymatic reactions, 10 μ L of 7.5 M guanidine hydrochloride was added, followed by 180 μ L of buffer (20 mM Tris, pH 8.0), mixed and then transferred to a 96-well FlashPlate (cat. no. SMP103; PerkinElmer). After mixing, the reaction mixtures in Flash plates were incubated for 1 h, and the CPM were measured using Topcount plate reader (PerkinElmer). The CPM counts in the absence of compound for each data set were defined as 100% activity. In the absence of the enzyme, the CPM counts in each data set were defined as background (0%). The IC₅₀ values were determined using GraphPad Prism 7 software.

For DOT1L, NSD1, NSD2, NSD3, ASH1L, DNMT3A/3L, and DNMT3B/3L, a filter-based assay was used. In this assay, 10 μ L of reaction mixtures were incubated at rt for 1 h, 50 μ L of 10% TCA was added, mixed, and transferred to filter-plates (Millipore; cat. no. MSFBN6B10; www.millipore.com). Plates were centrifuged at 2000 rpm (Allegra X-15R - Beckman Coulter, Inc.) for 2 min, followed by 2 additional 10% TCA wash and one ethanol wash (180 μ L), followed by centrifugation. Plates were dried and 100 μ L MicroO (MicroScint-O; cat. no. 6013611, PerkinElmer) was added to each well, centrifuged, and removed. 70 μ L of MicroO was added again, and CPM was measured using Topcount plate reader.

hNNMT Protein Expression and Purification for ITC and Crystallography

A construct encoding the full-length human NNMT was cloned into the pET28MHL expression vector using NdeI and HindIII restriction sites. Full-length human NNMT triple mutant (K100A:E101A:E103A) used for crystallization was ordered from the nonprofit plasmid repository addgene (Plasmid #40734). The recombinant hNNMT and hNNMTtm (triple mutant) were overexpressed in *E. coli* BL21 (DE3) codon plus RIL strain (Stratagene) by addition of 0.3 mM isopropyl-1-thio-D-galactopyranoside with a supplement of 1 mM zinc sulfate and incubated overnight at 15 °C. Harvested cells were resuspended in 50 mM

sodium phosphate buffer, pH 7.5, supplemented with 0.5 mM sodium chloride and 5% glycerol, and lysed using a microfluidizer (Microfluidics) at 10,000 psi. After clarification of the crude extract by high-speed centrifugation, the lysate was loaded onto a 5 mL HiTrap chelating column (GE Healthcare) charged with Ni^{2+} . The column was washed, and the protein was eluted with 50 mM Tris buffer, pH 8.0, 250 mM sodium chloride, 250 mM imidazole and supplemented with 0.5 mM of TCEP. The protein was next purified on a Superdex 200 column (26/600; GE Healthcare) equilibrated with 50 mM Tris-HCl buffer, pH 8.0, and 100 mM sodium chloride, and the elution fractions were pooled and supplemented with 0.5 mM of TCEP. All purification steps were performed at 4 °C and in the presence of a protease inhibitor AEBSF (Goldbio). The proteins were concentrated to ~20 mg/mL, and hNNMTtm was supplemented with 5% glycerol. The size and the purity of the recombinant hNNMT and hNNMTtm proteins were checked by SDS-PAGE and mass spectroscopy.

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) measurements were performed at 25 °C using a MicroCal ITC200 Instrument (Malvern Instruments). hNNMT was diluted at 100–200 μM in ITC buffer [50 mM Tris pH 8.0, 100 mM NaCl] supplemented with 2% DMSO for the binding with the compounds. Compounds **6** and **7** were prepared in DMSO at 50 mM and diluted to 1 mM in ITC buffer with a final DMSO concentration of 2%. The cofactors SAM, SAH, and the substrate nicotinamide were diluted to 2 mM. For the binding of the substrate nicotinamide in the presence of SAH, the protein was mixed at a ratio of 1:4 for 1 h at 4 °C prior the measurement. Binding constants were calculated by fitting the data using the ITC data analysis module in Origin 7.0 (OriginLab Corp).

Crystallization, Data Collection, and Structure Determination

Purified hNNMTtm at 20 mg/mL was mixed with compound **6** at a molar ratio of 1:4 and crystallized using the hanging drop vapor diffusion method at 17 °C by mixing equal volumes of the protein solution with the reservoir solution. The hNNMTtm–**6** binary complex was crystallized in 1.5 ammonium sulfate, 0.1 M HEPES pH 7.2. The crystal was soaked in the corresponding mother liquor supplemented with 20% ethylene glycol as cryoprotectant before flash freezing in liquid nitrogen.

X-ray diffraction data were collected at 100 K at NE-CAT beamline 24-ID-E of Advanced Photon Source (APS) at Argonne National Laboratory. The data were processed with MOSFLM, SCALA, and other programs from the CCP4 suite.⁴⁷ The structure of hNNMTtm complex was solved by molecular replacement using PHASER⁴⁸ using the atomic model of the hNNMTtm (Protein Data Bank ID code 2IIP). The location of the bound molecule was determined from a $F_o - F_c$ difference electron density map. REFMAC⁴⁹ and phenix.refine^{50,51} were used for structure refinement. Graphic program COOT⁵² was used for model building and visualization. The overall assessment of model quality was performed using MolProbity.⁵³ Crystal diffraction data and refinement statistics for the structure are displayed in Supporting Information Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

NNMT	nicotinamide <i>N</i> -methyltransferase
SAM	<i>S</i> -5'-adenosyl-L-methionine
ROS	reactive oxygen species
NAD⁺	nicotinamide adenine dinucleotide
WAT	white adipose tissue
ASO	antisense oligonucleotide
hNNMT	human NNMT
SAH	<i>S</i> -5'-adenosyl-L-homocysteine
SAR	structure–activity relationship
SAHH	SAH hydrolase
ITC	isothermal titration calorimetry
DOT1L	disruptor of telomeric silencing 1-like
PRMT7	protein arginine methyltransferase 7
SMYD2	SET and MYND domain containing protein 2
BCDIN3D	BCDIN3 domain containing RNA methyltransferase
MOA	mechanism of action

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Figure 1.
Chemical structures of NNMT inhibitors.

Figure 2.
Design of the bisubstrate NNMT inhibitors.

Figure 3. Docking analysis of compound **6** in the binding sites of hNNMT. (A) Docking of compound **6** (green) in the hNNMT (cyan) structure (PDB 2IIP). H-bond interactions are shown in yellow dotted lines. Water molecules are illustrated as red balls. (B) Overlay of the docking model with the hNNMT (gray)–nicotinamide (magenta)–SAH (yellow) complex (PDB 3ROD).

Figure 4. Biochemical and biophysical characterization of **6** and **7** and selectivity assessment of **6**. (A) IC₅₀ determination for **6** ($14 \pm 1.5 \mu\text{M}$; HS: 1.1) and **7** ($160 \pm 1 \mu\text{M}$) against hNNMT ($n = 3$). (B) ITC was used to assess binding of hNNMT to **6** (left) and **7** (right). (C) Selectivity of **6** was determined against a panel of 34 MTs and 1 HAT at two compound concentrations (10 μM (blue) and 50 μM (red)). (D) IC₅₀ determination for **6** against DOT1L ($1.3 \pm 0.2 \mu\text{M}$; HS: 1.1), PRMT7 ($20 \pm 2 \mu\text{M}$; HS: 1.1), BCDIN3D ($40 \pm 2 \mu\text{M}$; HS: 1.3), and SMYD2 ($62 \pm 7 \mu\text{M}$; HS: 0.6) were performed in triplicate. HS: Hill slope.

Figure 5. MOA study of NNMT inhibition with **6**. (A) Lineweaver–Burk plot of $1/\text{rate}$ versus $1/[\text{SAM}]$ with varying concentrations of inhibitor **6** showing competitive inhibition and (B) Lineweaver–Burk plot of $1/\text{rate}$ versus $1/[\text{nicotinamide}]$ with varying concentrations of inhibitor **6** showing noncompetitive inhibition. Eight concentrations of inhibitor **6** (from 2 to $250 \mu\text{M}$) were used for both experiments: $2 \mu\text{M}$ ($\bullet$), $4 \mu\text{M}$ ($\circ$), $8 \mu\text{M}$ ($\blacktriangle$), $16 \mu\text{M}$ ($\triangle$), $31 \mu\text{M}$ ($\blacksquare$), $63 \mu\text{M}$ ($\square$), $125 \mu\text{M}$ ($\blacklozenge$), and $250 \mu\text{M}$ ($\circ$). ITC was used to assess binding of (C) SAM and (D) nicotinamide to hNNMT.

Figure 6.

X-ray crystal structures. (A) The X-ray crystal structure of a binary complex of **6** (green) and hNNMT (cyan) (PDB 6B1A). The surface of the protein is shown in gray. (B) Key interactions between **6** and hNNMT. H-bond interactions are shown in yellow dotted lines. Water molecules are illustrated as red balls. (C) Overlay of the crystal structure of the hNNMT (cyan)–**6** (green) binary complex (PDB 6B1A) with the crystal structure of hNNMT (not shown)–nicotinamide (magenta)–SAH (yellow) complex (PDB 3ROD). (D) Overlay of the crystal structure of the hNNMT (cyan)–**6** (green) binary complex (PDB 6B1A) with the crystal structure of monkey NNMT (not shown)–**1** (blue)–SAH (yellow) complex (PDB 5XVQ).

Scheme 1. Synthetic route for 6 and 7^a

^aReagents and conditions: (a) $\text{NaBH}(\text{OAc})_3$, AcOH , DCM , rt , 16 h, 61–71%; (b) K_2CO_3 , DMSO , H_2O_2 , 0°C to rt , 3 h; (c) HCl in dioxane, 0°C to rt , 4 h; then 2 drops of water, rt , 4 h, 46–57% over 2 steps.