Supporting Information for:

Development of a Suicide Inhibition Based Protein Labeling (SIBLing) Strategy for Nicotinamide N-methyltransferase

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MATERIALS AND METHODS:

Chemicals and Reagents. The plasmid pET28a-LIC, harboring the NNMT gene was purchased from Addgene (Addgene plasmid no. 40734). The MTase-Glo™ Methyltransferase Assay kit was purchased from Promega. NEBNext High-Fidelity 2 X PCR master mix was purchased from New England BioLabs Inc. N-methyl-4-chloropyridine (**1**) was purchased from eNovation Chemicals. 4-chloronicotinamide (**3**) was purchased from Matrix Scientific. 6-chloronicotinamide (**4**) and 4, 6-dichloronicotinamide (**5**) were purchased from Ark Pharm Inc. 4-chloro-3-ethynylpyridine (**6**) was purchased from Enamine. All other chemicals were purchased from Sigma.

Site-directed Mutagenesis. The pET28-NNMT vector was used as a template to generate three cysteine to alanine mutants (i.e., C159A, C165A and C159/165A). Primers used for site-directed mutagenesis are listed in Table S4. The double mutant (C159/165A) was prepared by using the C159A mutant as a template and the primers used to generate the C165A mutant. PCR reactions were performed under standard conditions using the respective forward and reverse primers along with NEBNext High-Fidelity 2 X PCR master mix. The PCR product was incubated with 10 units of DpnI for 2 h at 37 °C followed by transformation into chemically competent *E. coli* BL21 (DE3) cells. Single colonies were picked and grown overnight in LB media with 50 μg/mL kanamycin. The desired mutations were confirmed by DNA sequencing of the entire gene. Expression and purification of the mutants was performed as described for the wild-type enzyme (see below).

Expression and Purification of Recombinant NNMT. Recombinant human NNMT was purified as previously described.[1] Briefly, the expression vector was transformed into *E. coli* BL21 (DE3) cells and grown in 2 L of Terrific Broth (TB) containing 50 μg/mL of kanamycin at

37 °C to an OD of 0.6-0.8 before the induction of protein expression with 1 mM IPTG and incubation overnight at 16 °C. Cells were harvested by centrifugation at 5,000 rpm (Rotor F9S-4x1000Y from Sorvall™) for 15 min at 4 °C and resuspended in lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 5 mM imidazole, 2 mM β-mercaptoethanol and Pierce™ EDTA-free protease inhibitor tablets (Thermo Scientific)) before sonication at amplitude of 30 for 20 s, oscillating between 1 s on and 1 s off. The crude lysate was then centrifuged at 35,000 rpm (Rotor 70Ti from Beckman Coultar Life Sciences) for 30 min at 4 °C. The supernatant containing soluble NNMT was incubated with Ni-NTA resin (GE biosciences) that was pre-equilibrated with lysis buffer, for 1 h at 4 °C. The resin was washed twice with 50 mM Tris-HCl pH 7.6, 0.5 M NaCl and 5% glycerol, containing 25 mM, 50 mM and 75 mM imidazole. The protein was eluted with 250 mM of imidazole in the above buffer and dialyzed overnight at 4 °C in dialysis buffer (50 mM Tris pH 7.6, 300 mM NaCl, 5% glycerol, and 1 mM DTT) to remove imidazole. The purified protein was found to be ~ 99% pure as indicated by SDS-PAGE with a protein marker to monitor the molecular weight. The dialyzed fractions were flash frozen using liquid nitrogen and stored at -80 °C until further use. The Bradford assay was used to determine protein concentrations. Note that NNMT purifies as a mixture in which a small fraction retains the initiator N-formyl methionine as revealed by mass spectrometry of the intact proteins (see Figure S2).

Transfection of NNMT into HEK293T Cells. The human NNMT gene was amplified from the pET28-NNMT vector by PCR. PCR primers encoding a 5′ KpnI site (along with a FLAG tag) and a 3′ XhoI site were used to amplify the NNMT gene (Table S4). The PCR product was purified using a PCR purification kit from Biobasic. The purified product was then digested with KpnI and XhoI and run on an agarose gel. The DNA was isolated by gel purification and then

ligated to KpnI and XhoI treated pcDNA 3.1 using T4 DNA ligase. HEK293T cells (ATCC) were transfected with the pcDNA3.1-NNMT construct using Lipofectamine 2000 according to the manufacturer's protocol. Stable transfection was established using hygromycin (Cellgro) as a selection marker. Briefly, HEK293T cells were seeded onto a tissue culture flask 1 day prior to transfection. Upon reaching 70-80% confluence, cells were transfected with 20 μg of pcDNA3.1-NNMT with Lipofectamine 2000. Cells were cultured for 48 h post-transfection and then selected with 0.15 mg/mL hygromycin. NNMT overexpression was confirmed by Western blotting.

Luminescence Assay to Monitor NNMT Activity. The MTase-Glo^{TM} Methyltransferase Assay Kit from Promega was used to monitor the activity of wild-type NNMT and the three cysteine mutants (i.e., the C159A, C165A and C159/165A mutants). In this assay, SAH formation (the by-product of the NNMT reaction) is measured as a function of time. Reactions were conducted in 20 mM Tris-HCl pH 8.6, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 0.1 mg/mL BSA and 0.5 mM TCEP at 25 ºC. Serial dilutions of SAH (0-10 μM) were used as a standard to convert the luminescence signal into concentration. Briefly, varying concentrations of NAM and SAM were incubated with NNMT (300 nM) for 15 min. The reaction was then quenched by addition of 0.1% TFA followed by addition of the MTase-Glo^{TM} Reagent to a final concentration of 1X. After a 30 min incubation at 25 ºC, the MTaseGlo^{TM} Detection Solution was added and the samples were incubated for a further 30 min at 25 ºC. Luminescence was then acquired with a PerkinElmer EnVision 2104 Multilabel Reader (λ_{ex} = 380 nm, λ_{em} = 530 nm). The kinetic parameters for NAM and SAM for wild-type NNMT and the mutants were determined by fitting initial rates obtained from these assays to equation 1,

$$\nu = V_{max}[S]/ K_M + [S] \quad (1),$$

using the GraphPad Prism 7.0 software.

Inactivation kinetics. The time-dependent kinetics of NNMT inactivation were determined by incubating NNMT (2 μM) in an inactivation mixture (20 mM Tris-HCl pH 8.6, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 0.1 mg/mL BSA and 0.5 mM TCEP, with a final volume of 80 μL) containing various concentrations of inhibitors (0-1.6 mM) at 25 ºC. Aliquots (10 μL) of the inactivation mixture were removed at various time points and were added to 90 μL of assay buffer containing 20 mM Tris-HCl pH 8.6, 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 0.1 mg/mL BSA, 0.5 mM TCEP, 50 μM NAM and 100 μM SAM with a final volume of 100 μL. After 20 min, the reactions were frozen with liquid nitrogen. Samples were thawed after completion of the time course and 0.1% TFA was added to quench the reactions followed by the addition of the MTase-Glo™ Reagent to a final concentration of 1X. After a 30 min incubation at 25 ºC, the MTaseGlo™ Detection Solution was added and the samples were incubated for a further 30 min at 25 ºC. SAH production was then quantified by measuring the luminescence. The time-dependence of NNMT inhibition was fit to equation 2,

$$\nu = \nu_0 \, e^{-kt} \qquad (2),$$

using Grafit, version 5.0.11, where ν is velocity, ν_0 is initial velocity, k (or k_{obs}) is the pseudo-first-order rate constant of inactivation, and t is the time. Since the plot of k_{obs} versus [I] did not saturate and was linear, k_{inact}/K_I was determined from the slope of the k_{obs} versus [I] plot. All the experiments were carried out at least in duplicate.

IC_{50} Studies. IC_{50} values were determined for both wild-type NNMT and the three cysteine mutants (i.e., C159A, C165A and C159/165A). The buffer conditions were the same as those described above. The concentrations of SAM (100 μM) and NAM (50 μM) were fixed. NNMT

(300 nM) with varying concentrations of inhibitor (0-320 μM) was incubated with NAM for 15 min at 25 ºC prior to initiation of the reaction by the addition of SAM. The reaction was quenched by 0.1% TFA after 20 min. After addition of the MTase-Glo™ Reagent and MTaseGlo™ Detection Solution (described earlier), SAH production was quantified by measuring the luminescence. IC_{50} values were determined by fitting the data to equation 3,

$$\text{Percent Activity of NNMT} = 1/ (1+ [I]/IC_{50}) \quad (3),$$

where [I] is the concentration of inhibitor and IC_{50} is the concentration of inhibitor at half the maximum enzymatic activity.

Dialysis Experiment. NNMT inactivated by **1** was subjected to extensive dialysis in 50 mM Tris (pH 7.6) overnight (16 h) at 4 ºC and the recovery of enzyme activity was measured using the luminescence assay.

Fluorescence Assay to Monitor NNMT Inhibition. A previously described[2] continuous fluorescence assay was applied to measure NNMT enzymatic activity in reaction conditions where there was a chance of interference from residual SAH. This assay uses quinoline as an alternative NNMT substrate and generates fluorescent 1-methyl quinolinium (MQ). The assay buffer contained 5 mM Tris pH 8.6 and 0.5 mM TCEP. NNMT (2 μM) was incubated with compounds **2, 3** and **6** (0.5 mM) in the presence of SAM (0.5 mM) for 0.5 h at 25 ºC and then unreacted small molecules (i.e., substrate/inhibitor, SAM and SAH) were removed from the reaction mixture using a desalting column. The concentration of the modified enzyme was determined by Bradford assay. The modified enzyme (300 nM) was then added to quinoline (20 μM) and the reactions were initiated in a 96-well plate (Corning® Half Area Black Flat Bottom Polystrene NBSTM, product #3993) by the addition of SAM (100 μM). Serial dilutions of MQ

(0-30 μM) were used as a standard to convert the fluorescence signal into concentration. MQ fluorescence (λ_{ex} = 330 nm, λ_{em} = 405 nm) was detected in real time at 20 sec intervals for 15 min using a PerkinElmer EnVision 2104 Multilabel Reader in conjunction with Wallac EnVision Manager software and enzyme activity was determined from the initial rates.

Fluorescence Assay to Measure Time-Dependent NNMT Inactivation. To measure the time-dependent inactivation of NNMT, the enzyme was incubated with its suicide inhibitors (0.5 mM) in the presence of SAM (0.5 mM) at 25 ºC. Aliquots (20 μL) of the inactivation mixture were removed at various time points followed by the desalting process to remove the unreacted compounds. The activity of the modified enzyme was then determined using the fluorescence assay and the inactivation kinetics were evaluated using equation 2 as already described.

LC-MS Analysis of Inactivated NNMT. The covalent adducts formed during NNMT inactivation by either **1** or by the suicide inhibitors **2**, **3** and **6** in presence of SAM, were characterized by an Agilent HPLC coupled to ESI-MS mass spectrometer. NNMT (0.5 mg/ml) was incubated with the inactivators (100 μM) and SAM (100 μM) in 5 mM HEPES (pH 7.6) for 30 min at 25 ºC. The unreacted small molecules were removed using a Centricon centrifugal filter device with a 3000 Da molecular weight cutoff (MWCO) and 100 μL of the protein sample was loaded onto the mass spectrometer. A control sample without inhibitor was prepared in the same way.

Dose-dependent *In Vitro* Labeling of NNMT with 6. Pure NNMT (1 μM) plus 50 μM SAM were incubated with increasing concentrations of 4-chloro-3-ethynylpyridine (**6**) in 1x PBS (pH 7.6) at 37 ºC for 1 h. Labeled enzyme was then coupled to TAMRA-N_3 (50 μM) in the presence of 1X TBTA (0.31 mM), TCEP (1 mM) and freshly prepared $CuSO_4$ (1 mM). The tubes were

gently tumbled for 2 h. The reactions were then quenched by adding 6x-SDS loading buffer and separated by SDS-PAGE (4-20% gel). The bands were visualized by scanning the gel in a typhoon scanner (approximate excitation/emission maxima ~546/579, respectively).

Limit of Detection for *In Vitro* Labeling of NNMT with 6. Compound **6** (25 μM) along with 50 μM SAM were incubated with decreasing concentrations of recombinant NNMT (1.0 to 0.1 μg) in 1x PBS (pH 7.6) at 37 ºC for 1 h. The probe labeled enzymes were coupled to TAMRA-N_3 (50 μM) in the presence of 1X TBTA (0.31 mM), TCEP (1 mM) and freshly prepared $CuSO_4$ (1 mM). The tubes were gently tumbled for 2 h. The reactions were then quenched by adding 6x SDS loading buffer and separated by SDS-PAGE (4-20% gel). The bands were visualized by scanning the gel in a typhoon scanner (approximate excitation/emission maxima ~546/579, respectively).

Labeling of NNMT with 6 in HEK293TNNMT and SKOV-3 Cell Lysates. HEK293T cells stably expressing human NNMT (HEK293TNNMT) and Ovarian Carcinoma SKOV-3 Cells (ATCC) that endogenously expresses NNMT were cultured in Corning® 1X DMEM (Dulbecco's Modified Eagle's Medium) with L-glutamine, 10% heat-inactivated FBS and 1 X Corning® Penicillin-Streptomycin solution. Cells were grown to ~80% confluence (6 x 10^7 cells), then trypsinized, and trypsin activity quenched with complete media. Cells were then harvested by centrifugation at 3,500 rpm for 3 min. Next, the cells were washed with 1X PBS four times and were resuspended in 1X PBS at 5 x 10^6 cells/mL and 4 x 10^5 cells were used for subsequent assays. HEK293T-NNMT and SKOV-3 cells were lysed by sonication at 4 ºC with a micro-tip probe sonicator at an amplitude of 10 for 20 s, oscillating between 1 s on and 1 s off. After centrifugation at 21,000 x g for 20 min, lysates were collected, and the soluble protein fraction was quantified by the Detergent Compatible assay (DC assay, Biorad). HEK293T-

NNMT cell lysates (2 μg/μL, 80 μg total) spiked with 0.5 μg of recombinant NNMT and SKOV-3 cell lysates (2 μg/μL, 80 μg total) were treated with increasing concentrations of **6** (0 to 50 μM) in the presence of SAM (50 μM) and the cells were incubated for 2 h followed by "Click" reaction with TAMRA-N_3 (50 μM) in presence of 1X TBTA (0.31 mM), TCEP (1 mM) and freshly prepared $CuSO_4$ (1 mM). The tubes were gently tumbled for 2 h. The reactions were then quenched by adding 6x-SDS loading buffer lacking beta-mercaptoethanol and proteins were separated by SDS-PAGE (4-20% gel). The bands were visualized by scanning the gel in a typhoon scanner (approximate excitation/emission maxima ~546/579, respectively).

Cellular Labeling of NNMT with 6 in HEK293TNNMT and SKOV-3 Cell Lysates. HEK293T-NNMT and SKOV-3 cells, grown to ~80% confluence (1 x 10^7 cells) in 6-well plates, were treated with increasing concentrations of **6** (0 to 50 μM) in the presence of SAM (50 μM) and the cells were incubated in serum free media for 2 h. Cells were then trypsinized and trypsin activity quenched with complete media. Cells were then harvested by centrifugation at 1,000 x g for 3 min. Next, the cells were washed with 1X PBS four times and were resuspended in 1X PBS followed by sonication and centrifugation. Lysates were collected and the soluble protein fraction was quantified by the DC assay. Lysates (2 μg/μL, 80 μg total) were "Clicked" with TAMRA-N_3 (50 μM) in presence of 1X TBTA (0.31 mM), TCEP (1 mM) and freshly prepared CuSO4 (1 mM). The tubes were gently tumbled for 2 h. The reactions were then quenched by adding 6x SDS loading buffer lacking beta-mercaptoethanol and separated by SDS-PAGE (4-20% gel). The bands were visualized by scanning the gel in a typhoon scanner (approximate excitation/emission maxima ~546/579, respectively).

Cellular Inhibition of NNMT by 2, 3 and 6 in HEK293TNNMT Cells. HEK293T-NNMT cells, grown to ~80% confluence (1 x 10^7 cells) in 6-well plates, were treated with increasing

concentrations of **2**, **3** and **6** (0 to 400 μM) in the presence of SAM (200 μM) and the cells were incubated for 2 h. Cells were trypsinized and then trypsin activity was quenched with complete media and cells were harvested by centrifugation at 1,000 x g for 3 min. Next, the cells were washed with 1X PBS four times and were resuspended in 1X PBS followed by sonication and centrifugation. Lysates were collected and the soluble protein fraction was quantified by the DC assay. The activity of the modified enzyme was determined using the quinoline assay as described earlier.

Determination of Partition Ratio of Recombinant NNMT to 6. Recombinant NNMT (2 μM) was incubated with varying concentrations (0-0.4 mM) of **6** in the presence of SAM (0.5 mM) for 2 h at 25 ºC to ensure that the inactivation had proceeded to the maximum extent. Unreacted small molecules were then removed from the reaction mixture using PD SpinTrap^{TM} G-25 sephadex columns (GE Healthcare) and the activity of the modified enzyme was determined using the quinoline assay as described earlier. The fractional enzyme activity was then plotted against [I]/[E] and the turnover number (r + 1) is determined from the intercept of the linear regression line on X-axis.

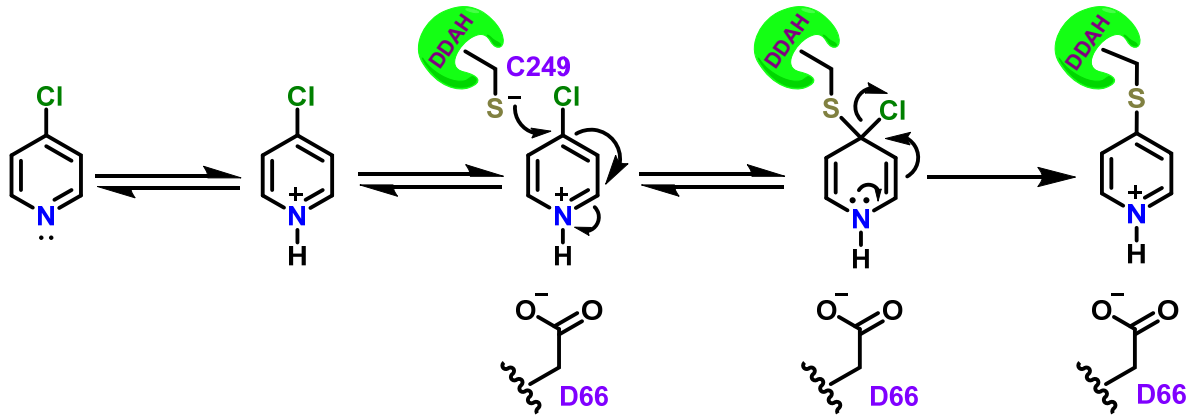

Figure S1: Nucleophilic aromatic substitution of 4-chloropyridine by DDAH.

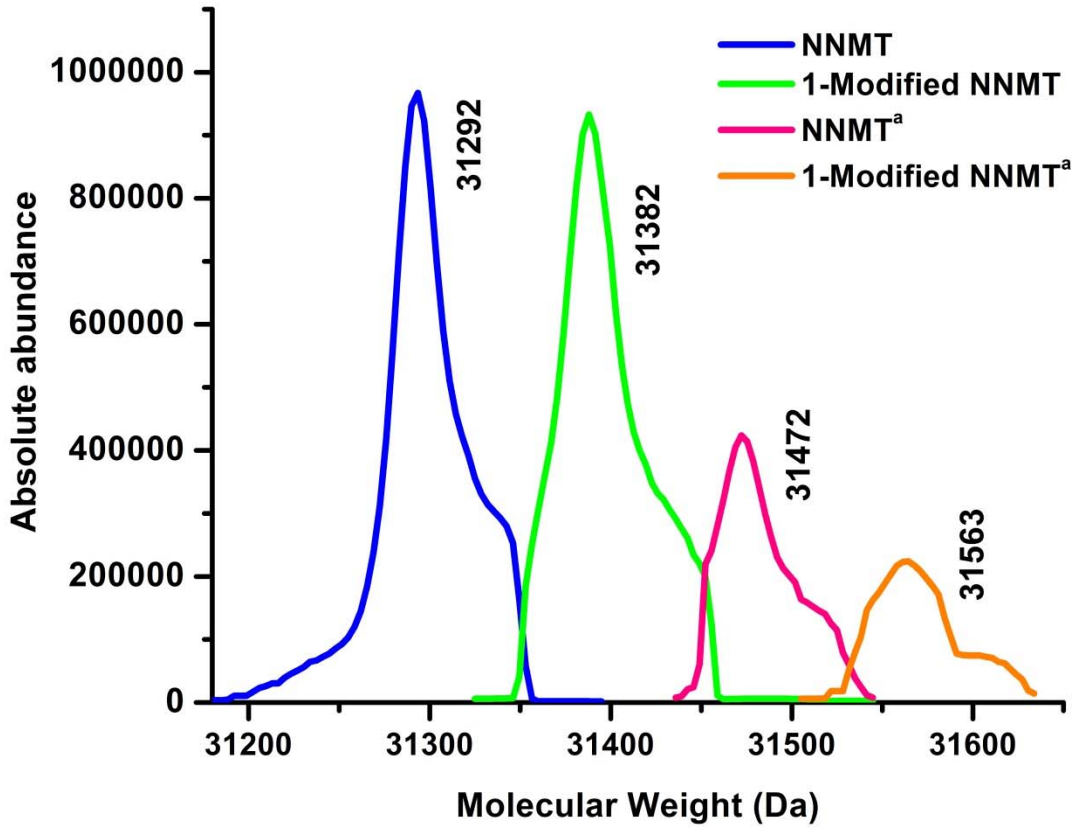

Figure S2: Reaction between wild type NNMT and compound **1** in the presence of SAM as indicated by the change in mass as detected by LC-ESI-MS. The peaks correspond to the deconvoluted molecular weights for unmodified NNMT and NNMT N-formyl methionine (represented by NNMT[a]), **1**-modified NNMT and **1**-modified NNMT[a].

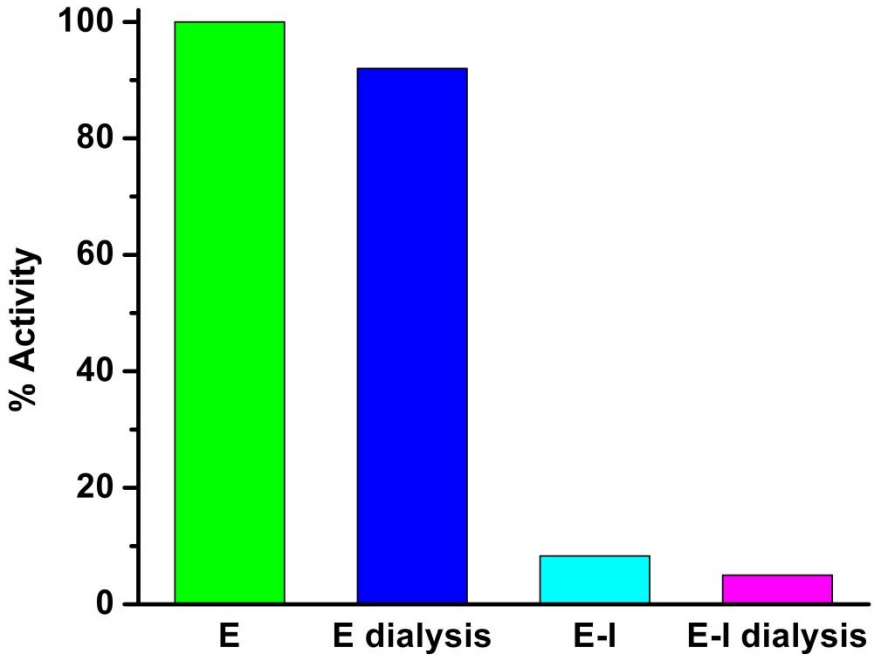

Figure S3: Enzyme inactivated by **1** was dialyzed overnight and activity was measured. "E" is the enzyme- control; "E-dialysis" is enzyme after dialysis; "E-I" is inactivated enzyme by **1**; "E-I dialysis" is inactivated enzyme after dialysis.

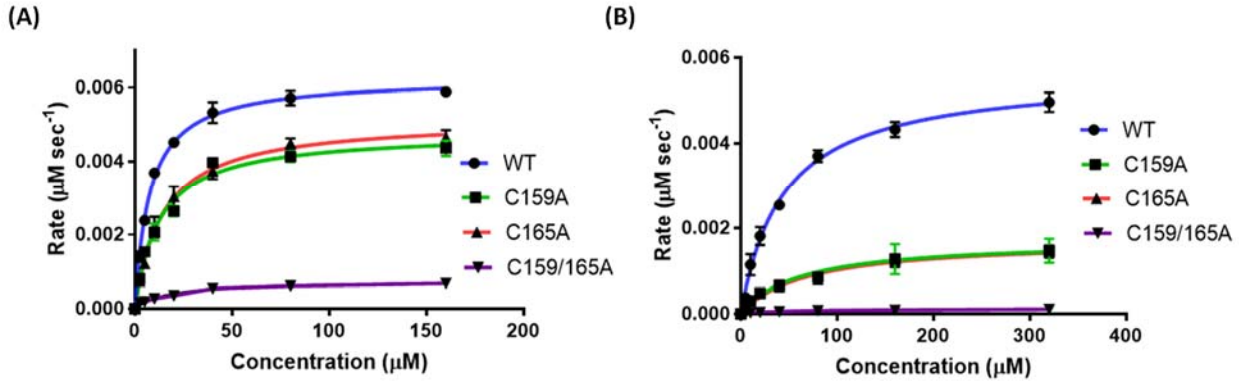

Figure S4: NNMT and the C159A, C165A and C159/165A mutants methylate **(A)** NAM and **(B)** compound **2**. All experiments were performed in duplicate (n = 2).

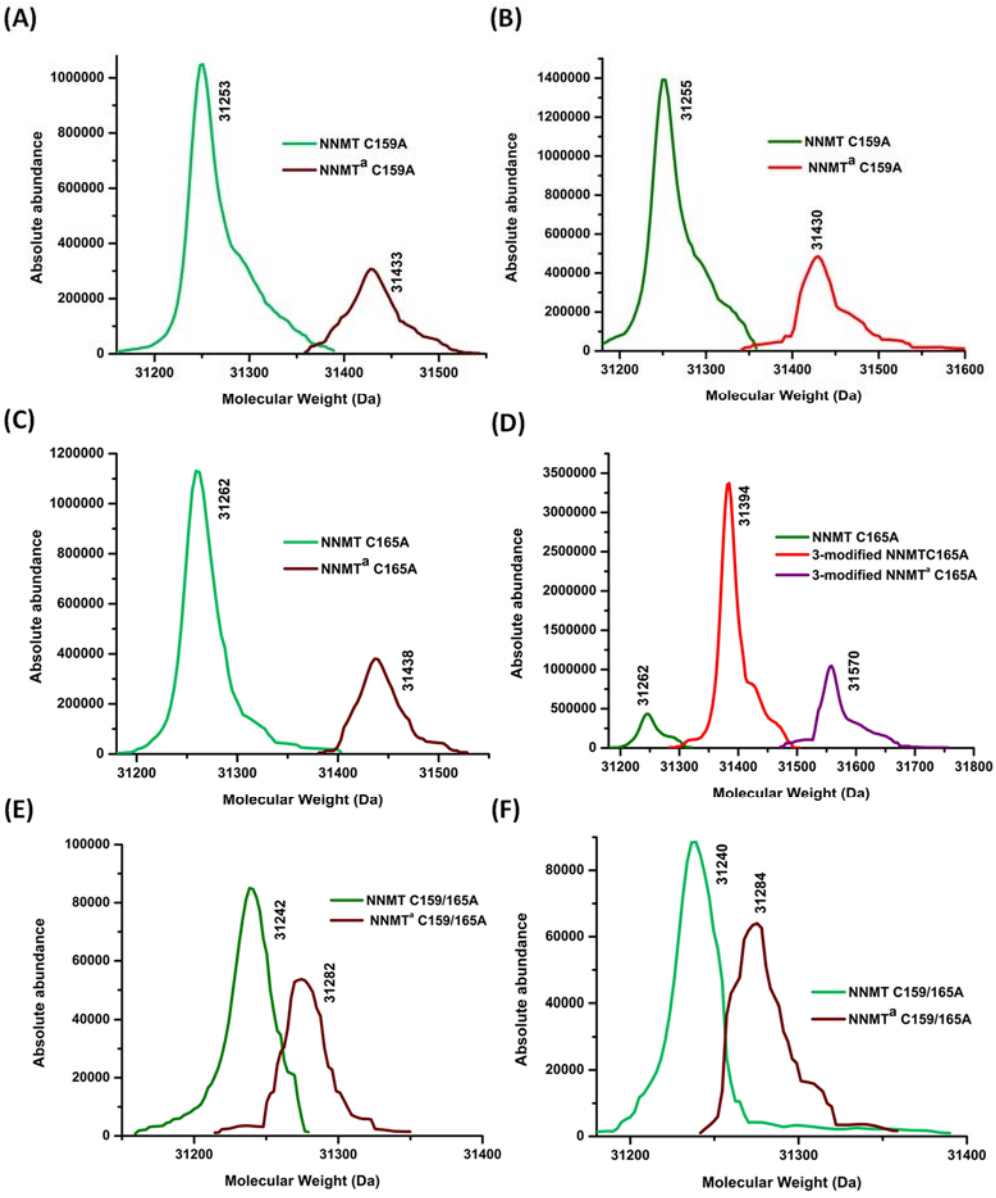

Figure S5: The deconvoluted mass spectra for unmodified NNMT and NNMT containing an uncleaved N-formyl-methionine (NNMT[a]) for **(A)** the NNMTC159A mutant, **(B)** the NNMT C159A mutant in presence of 3 and SAM, **(C)** the NNMTC165A mutant, **(D)** the NNMTC165A mutant incubated with 3 and SAM, **(E)** the NNMTC159/165A mutant, and **(F)** the NNMTC159/165A mutant in presence of 3 and SAM.

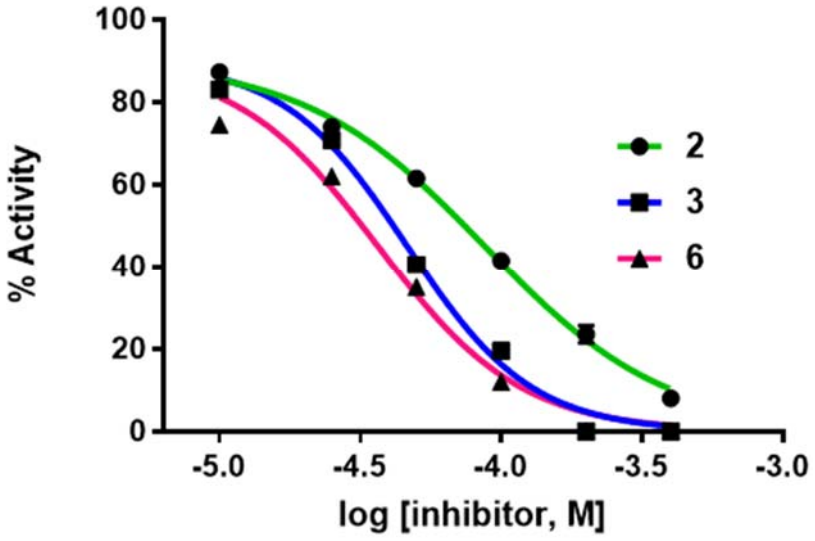

Figure S6: Inhibition of NNMT by **2**, **3** and **6** in HEK293NNMT Cells. Cells were treated with increasing concentrations of inhibitors (0-400 μM) in presence of SAM for 2 h**.** Cells were then isolated and washed with PBS to remove the unreacted compounds and lysed. The activity of NNMT was measured by monitoring the methylation of quinoline; the product, 1-methyl quinolinium, is fluorescent ((λ_{ex} = 330 nm, λ_{em} = 405 nm). The EC_{50} values for **2**, **3** and **6** were 87±2 μM, 47±1 μM and 36±1 μM respectively. All experiments were performed in triplicate (n = 3)

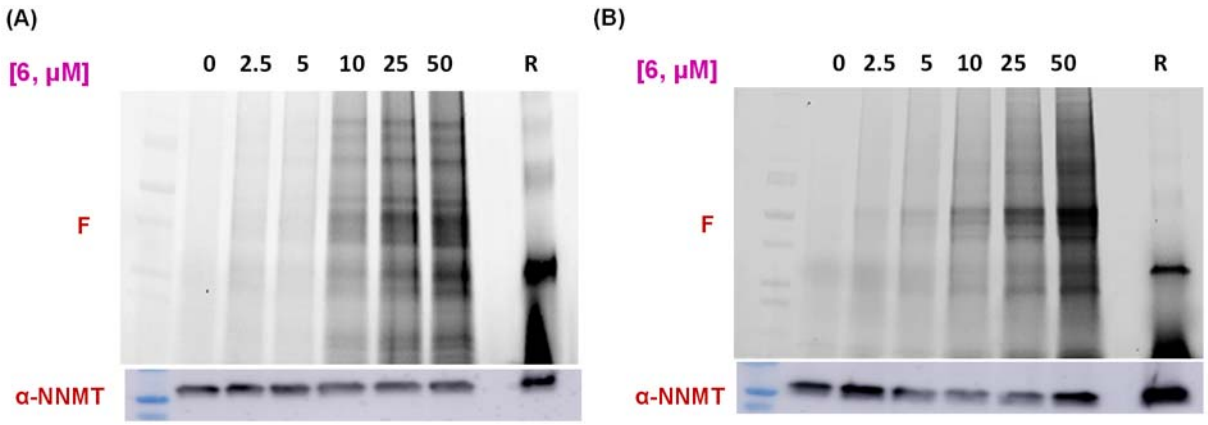

Figure S7: (A) Concentration-dependent labeling of HEK293NNMT cells by **6** in the presence of SAM**.** F and R stand for fluorograph and recombinant NNMT (B) Concentration dependent labeling of SKOV3 cells by **6** in the presence of SAM**.** Both experiments were performed in triplicate (n = 3)

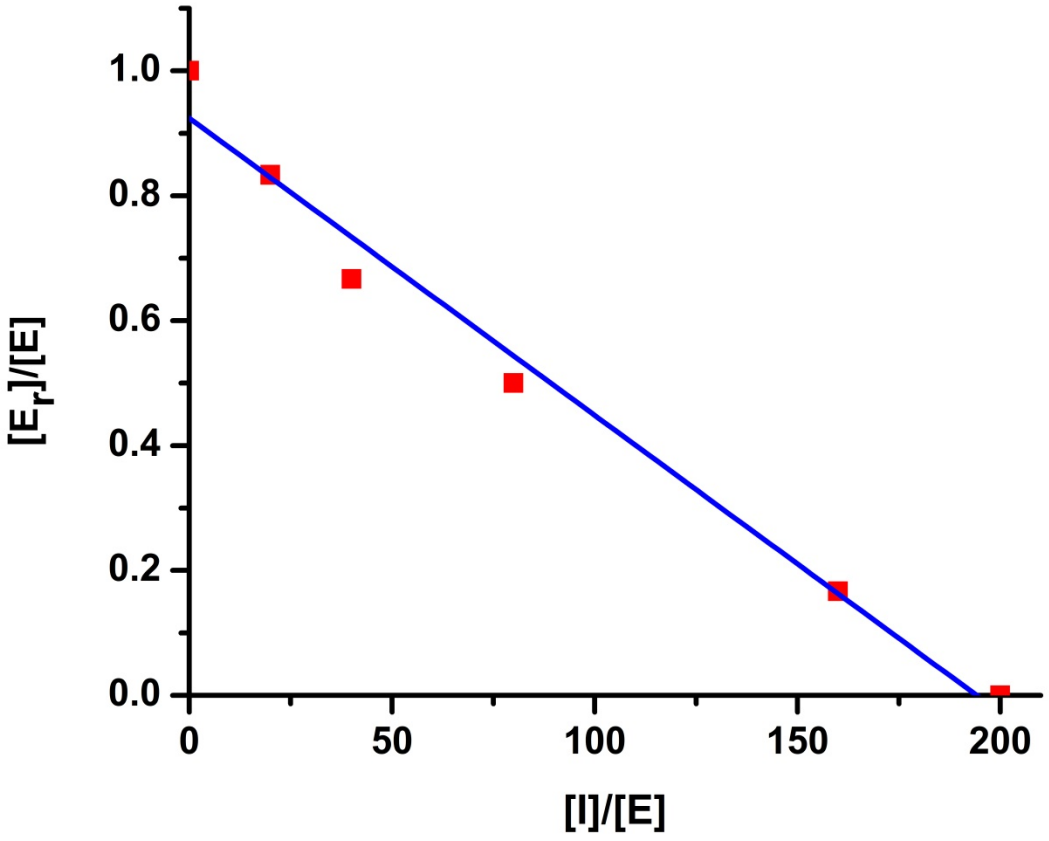

Figure S8: Determination of the partition ratio (*r*) for the inactivation of NNMT by 6. Recombinant NNMT (2 μM) was incubated with increasing concentrations of **6** (0-0.4 mM) in presence of SAM and enzyme activity was measured by monitoring the methylation of quinoline; the product, 1-methyl quinolinium, is fluorescent ((λ_{ex} = 330 nm, λ_{em} = 405 nm). The partition ratio was ~ 200 (R^2 = 0.98). This experiment was performed in triplicate (n = 3).

Table S1: Kinetic parameters for the methylation of NAM, 2, 3 and 6 by wild-type NNMT and mutant NNMT.

NNMT	NNMT Substrates							
	NAM		**2**		**3**		**6**	
	k_{cat} (s^{-1})	K_M (μM)	k_{cat} (s^{-1})	K_M (μM)	k_{cat} (s^{-1})	K_M (μM)	k_{cat} (s^{-1})	K_M (μM)
WT	0.02±0.007	7±0.2	0.02±0.003	44±2	0.02±0.005	22±0.9	0.01±0.002	23±2
C159A	0.02±0.003	12±1	0.003±0.0007	61±7	0.003±0.0001	37±1	ND[a]	ND[a]
C165A	0.02±0.001	14±1	0.003±0.0005	67±9	0.003±0.0003	35±2	ND[a]	ND[a]
C159/ 165A	0.002±0.0002	16±2	0.0003±0.00004	43±3	0.0003±0.00005	36±2	ND[a]	ND[a]

[a]ND = Not determined.

Table S2: The deconvoluted molecular weights of unmodified and modified NNMT.

Type		Mass (Da)		Observed Change (Da)	Expected Change (Da)
		Unmodified	Modified		
WT	NNMT	31294	ND[b]	0	0
	NNMT[a]	31472			
WT+2+SAM	NNMT	31292	31382	90 (1)[c]	92
	NNMT[a]	31470			
WT+3+SAM	NNMT	ND[b]	31428	134 (1)[c]	135
			31558	264 (2)[c]	
	NNMT[a]		31602	130 (1)[c]	

[a] NNMT in which the N-formyl methionine has not been removed from the enzyme.

[b]ND = Not detected.

[c] Number of residues being modified is shown within parentheses.

Table S3: The deconvoluted molecular weights of unmodified and modified NNMT Mutants.

NNMT Mutant		Mass (Da)		Observed Change (Da)	Expected Change (Da)
		Unmodified	Modified		
C159A	NNMT	31253	ND[b]	0	0
	NNMT[a]	31433			
C159A+3+ SAM	NNMT	31255	ND[b]	0	135
	NNMT[a]	31430			
C165A	NNMT	31262	ND[b]	0	0
	NNMT[a]	31438			
C165A+3+ SAM	NNMT	31262	31394	132 (1)[c]	135
	NNMT[a]		31570	132 (1)[c]	135
C159/165A	NNMT	31242	ND[b]	0	0
	NNMT[a]	31282			
C159/165A+ 3+SAM	NNMT	31240	ND[b]	0	135
	NNMT[a]	31284			

[a] NNMT in which the N-formyl methionine has not removed from the enzyme.

[b]ND = Not detected.

[c] Number of residues being modified is shown within parentheses.

Table S4: Primers for Mutagenesis and Cloning.

C159A	Forward 5′ - CCGGCTGACGCTGTGCTCAGCACACTG - 3′ Reverse 5′ - CAGTGTGCTGAGCACAGCGTCAGCCGG - 3′
C165A	Forward 5′- GTGCTCAGCACACTGGCACTGGATGCCGCCTG - 3′ Reverse 5′- CAGGCGGCATCCAGTGCCAGTGTGCTGAGCAC - 3′
Cloning	Forward 5′- CATGGTACCATGGACTACAAGGACGACGACGACAAGATGG AATCAGGCTTCACC - 3′ Reverse 5′ - GTCTCGAGTCACAGGGGTCTGCTCAGCTT - 3′

REFERENCES

(1) Loring, H. S.; Thompson, P. R. Kinetic Mechanism of Nicotinamide N-Methyltransferase. *Biochemistry* **2018**, *57*, 5524.

(2) Neelakantan, H.; Vance, V.; Wang, H. L.; McHardy, S. F.; Watowich, S. J. Noncoupled Fluorescent Assay for Direct Real-Time Monitoring of Nicotinamide N-Methyltransferase Activity. *Biochemistry* **2017**, *56*, 824.