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

Sudeshna Sen†‡, Santanu Mondal†‡, Li Zheng†‡, Ari J. Salinger†‡, Walter Fast⸸, Eranthie Weerapana§ and Paul R. Thompson\*†‡

†Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, Massachusetts 01605, United States.

‡ Program in Chemical Biology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, Massachusetts 01605, United States.

⸸ Division of Chemical Biology and Medicinal Chemistry, College of Pharmacy, University of Texas, Austin, Texas 78712, United States.

§ Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02467, United States.

\*Author to whom correspondence should be addressed: Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, LRB 826, 364

Plantation Street,

Worcester, MA 01605 tel: 508-856-8492; fax: 508-856-6215; e-mail:

paul.thompson@umassmed.edu.

**ABSTRACT**

Nicotinamide N-methytransferase (NNMT) catalyzes the S-adenosyl-L-methionine (SAM)-dependent methylation of nicotinamide (NAM) to form N-methylnicotinamide (Me-NAM). This enzyme detoxifies xenobiotics and regulates NAD+ biosynthesis. Additionally, NNMT is overexpressed in various cancers. Herein, we describe the first NNMT-targeted suicide substrates. These compounds, which include 4-chloropyridine and 4-chloronicotinamide, exploit the broad substrate scope of NNMT; methylation of the pyridine nitrogen enhances the electrophilicity of the C4 position, thereby promoting an aromatic nucleophilic substitution by C159, a non-catalytic cysteine. Based on this activity, we developed a suicide inhibition-based protein labeling (SIBLing) strategy using an alkyne-substituted 4-chloropyridine that selectively labels NNMT *in vitro* and in cells. In total, this study describes the first NNMT-directed activity-based probes.

Nicotinamide N-methyltransferase (NNMT) is a cytosolic S-adenosyl-L-methionine (SAM)-dependent methyltransferase that catalyzes the methylation of nicotinamide (NAM) to form N-methylnicotinamide (Me-NAM) and S-adenosyl-L-homocysteine (SAH) as a by-product (Figure 1A).[1](#_ENREF_1), [2](#_ENREF_2) NNMT also methylates numerous other pyridine-containing compounds.[3](#_ENREF_3) Although widely expressed, NNMT is predominantly found in the liver, where it detoxifies xenobiotics by producing N-methylated metabolites.[1-9](#_ENREF_1) Additionally, NNMT regulates NAD+ biosynthesis.[8](#_ENREF_8), [10](#_ENREF_10) Overexpression of NNMT is linked with several diseases, including Parkinson’s disease where it has been proposed to generate N-methylated pyridiniums that are dopaminergic toxins,[11](#_ENREF_11), [12](#_ENREF_12) although this idea is controversial.[3](#_ENREF_3), [13](#_ENREF_13) NNMT also regulates adiposity by altering intracellular NAD+ and SAM levels. Notably, higher NNMT levels are observed in the white adipose tissue of mice suffering from obesity and type 2 diabetes.[14](#_ENREF_14) Interestingly, NNMT knockdown increases energy expenditure and prevents weight gain in mice receiving a fat-enriched diet. These data clearly indicate a relationship between NNMT and obesity.[8](#_ENREF_8) Finally, increased NNMT activity alters the intracellular levels of SAM, which interferes with the methylation of DNA and histones and thereby modulates gene expression.[15](#_ENREF_15) This effect ultimately impacts a variety of cancers of the lung,[16](#_ENREF_16) thyroid,[17](#_ENREF_17), [18](#_ENREF_18) brain,[19](#_ENREF_19) kidney,[20](#_ENREF_20) bladder,[19](#_ENREF_19) pancreas, and stomach.[21](#_ENREF_21) Notably, NNMT knockdown decreases cell migration, proliferation, and metastasis.

Given the therapeutic potential of NNMT, there is increasing interest in developing NNMT inhibitors. NNMT is a small 29 kDa globular protein that is structurally related to other Class-I SAM-dependent methyltransferases.[22](#_ENREF_22) Structures of NNMT bound to NAM and SAH have been determined and are consistent with the direct transfer of a methyl group to the pyridine nitrogen.[22](#_ENREF_22) Recently, we showed that human NNMT uses a rapid equilibrium ordered mechanism where SAM binds first followed by NAM. Methyltransfer occurs at which point Me-NAM is released, followed by SAH.[23](#_ENREF_23) A limited number of NNMT inhibitors have been reported. For example, the product inhibitors Me-NAM and SAH are reasonably potent (*K*i (MeNAM) = 27 µM and *K*i (SAH) =3 µM, respectively) reversible inhibitors that we used to determine the kinetic mechanism of NNMT (see above). Several quinolines are also reversible inhibitors.[24](#_ENREF_24) Recently, bisubstrate inhibitors (IC50 ~ 20 µM) were developed by conjugating NAM and SAM through an alkyl linker.[25](#_ENREF_25), [26](#_ENREF_26) 6-methoxynicotinamide, another reversible NNMT inhibitor (IC50 = 1.8 µM) reduces Me-NAM levels and body weight gain in mouse models of metabolic diseases.[27](#_ENREF_27) Cravatt and colleagues also described the first irreversible NNMT inhibitor (*k*obs/[I] = 21 M-1s-1). This compound uses a cysteine-targeted α-chloroacetamide warhead and covalently modifies C165, a non-catalytic cysteine present in the active site.[28](#_ENREF_28)

Fast reported that 4-chloropyridines (Figure 1B) covalently modify the active-site cysteine in dimethylarginine dimethyliminohydrolase (DDAH), an unrelated enzyme.[29-32](#_ENREF_29) While the neutral form of 4-choloropyridine was unreactive, protonation of the pyridine ring nitrogen by the enzyme enhanced the electrophilicity of the 4-chloro position (Figure S1). N-methylation mimicked this effect. Given the presence of cysteines in the active-site of NNMT, we questioned whether NNMT might accept halogenated pyridines as substrates to generate its own inhibitor (Figure 1C). A major advantage of this “suicide inhibition” strategy is reduced off-target toxicity since NNMT is generating its own inhibitor.

To test this hypothesis, we first determined whether N-methyl-4-chloropyridine (**1**, Figure 1B) inactivates NNMT. Here, we incubated purified NNMT with **1** and monitored the residual methyltransferase activity at different times by monitoring SAH production (Figure 1D). Gratifyingly, compound **1** exhibited time and concentration-dependent inhibition consistent with an irreversible inactivation mechanism. From the time-dependence data, we calculated a *k*inact/*K*I value of 44±3 M-1min-1 (Figure 1E), which is similar to that obtained for the inactivation of DDAH by 4-halopyridines. Next, we incubated **1** with NNMT and measured the intact mass of the protein. The 90 Da mass increase is consistent with the expected mass change of 92 Da for a singly modified protein (Figure S2). We confirmed irreversibility by measuring activity after dialysis; there was no recovery of activity consistent with the covalent inactivation of NNMT by **1** (Figure S3).

Having confirmed that N-methyl-4-chloropyridine irreversibly inhibits NNMT, we next determined whether 4-chloropyridine (**2**) is an NNMT substrate (Figure 1C) by measuring SAH production**.** Gratifyingly, the initial rate data exhibit Michaelis-Menten kinetics (Table S1) and the KM of **2** (KM(2) = 44 µM) is similar to that obtained for NAM (KM (NAM) = 7 µM). Next, we tested a small library of chloropyridines (compounds **3-5**, Figure 1B). Like **2**, NNMT methylates compound **3** (KM(3) = 23 µM) indicating that the 4-chloro substitution is well tolerated. By contrast, compounds **4** and **5** were not methylated suggesting that substitution of the 2-position is forbidden (Figure 2A).

We next investigated whether N-methylation facilitates enzyme inactivation by incubating **2** and **3** with NNMT and SAM for 0.5 h. Unreacted small molecules were removed with a desalting column and residual activity assayed by measuring quinoline methylation; 1-methyl-quinoline is fluorescent.[33](#_ENREF_33) Quinoline is a known NNMT substrate (KM(Q) = ~17 µM and *k*cat = ~ 0.75 min-1), which we used to determine the kinetic mechanism of NNMT.[23](#_ENREF_23) This fluorescence assay minimizes the interference from residual SAH that was not removed by the desalting column. Using this assay, we found that **2** and **3** inactivate NNMT similarly to compound **1** (Figure 2B). Moreover, both compounds exhibit time-dependent enzyme inactivation kinetics with *k*obs/[I] values of 20±1 M-1min-1 for **2** and 80±2.5 M-1min-1 for **3** (Figure 2C). The covalent modification of NNMT by **2** and **3** was confirmed by mass spectrometry. The observed mass shifts (90 and 134 Da) are consistent with those expected for NNMT modified by a single molecule of **2** (92 Da) or **3** (135 Da). Note, we did observe a small amount of NNMT modified by two molecules of **3** (Figure 2D-F, Table S2). Taken together, these data demonstrate that **2** and **3** are suicide inhibitors that rely on NNMT catalysis to generate the reactive species.

Since N-methyl-chloropyridines preferentially target cysteine, we next identified the cysteine that is modified. Based on the NNMT-NAM-SAH crystal structure (PDB ID: 3ROD), we identified two non-catalytic cysteines out of total 8 cysteine residues, C159 and C165, that were closer to the active site than the others (Figure 3A). Notably, Cravatt and colleagues showed that covalent modification of C165 inhibits NNMT activity.[28](#_ENREF_28) Given these data, we generated the C159A, C165A and C159/165A mutants to confirm whether enzyme inactivation is due to the covalent modification of one or both residues. The three mutants were purified and the steady state kinetic parameters were determined (Table S1). Although the *k*cat of the C159/165A double mutant is reduced by ~10 fold, neither single mutant showed any effect on this parameter. Moreover, there were minimal effects on *K*M. These results suggest that C159 and C165 are not important for substrate binding but instead act as second shell residues that indirectly influence catalysis.

Next, we showed that these mutants methylate **2** and **3** (Figures 3B, S4 and Table S1). Compared to wild-type NNMT, *k*cat is ~10-fold lower. The reason for the decreased rate of methylation of **2** and **3** is not immediately clear. Interestingly, the IC50 values of **1** for wild-type NNMT and the C165A mutant are similar. By contrast, little to no inhibition was observed for the C159A and C159/165A mutants (Figure 3C). Similarly, when we evaluated the ability of **2** and **3** to act as suicide inhibitors, we saw no inhibition of the C159A mutant (Figure 3D). By contrast, the C165A mutant remained sensitive. Unfortunately, we could not evaluate C159/165A NNMT mutant in this assay set-up as this mutant was inactive after passing through the desalting column. Nevertheless, these results unambiguously indicate that C159 is covalently modified by N-methyl-4-chloropyridine and N-methyl-4-chloronicotinamide. These observations are also consistent with the mass shifts obtained from MS studies (Figure S5, Table S3). The NNMT-NAM-SAH structure adopts a highly compact fold and C159 is not directly accessible from the active site. This data indicates that the during the catalytic cycle the enzyme must undergo a conformational change to either increase its accessibility and/or release the activated inhibitor such that it can rebind to NNMT adjacent to C159.

Building on these findings, we next developed a suicide inhibition-based protein labeling (SIBLing) strategy using compound **6** (Figure 1); **6** contains an alkyne that can be conjugated with a reporter tag via the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction after enzyme inactivation. Gratifyingly, compound **6** is methylated by NNMT and the kinetic values are similar to those obtained for **2**, **3,** and NAM (Table S1), thereby indicating that the alkyne does not impact the binding affinity of **6** (Figure 4A). Having established that **6** is also an NNMT substrate, we next treated NNMT with **6** and SAM. Notably, this treatment readily inactivated the enzyme (Figure 4B). The intact mass of the modified protein is consistent with the addition of a single molecule of **6** (Figure 4B, inset). Next, we determined whether **6**, as well as **2** and **3**, can inhibit cellular NNMT activity. For these studies, NNMT overexpressing HEK293T cells were treated with increasing concentrations of **2**, **3** and **6** for 2 h. Cells were lysed and NNMT activity was measured. The results showed that **2, 3** and **6** inhibit cellular NNMT activity with EC50 values in the double digit micro molar range respectively (Figure S6).

 Having established that **6** is a suicide inhibitor and can inhibit cellular NNMT activity, we next treated NNMT with a mixture of SAM and **6** and then used CuAAC to couple the alkyne to TAMRA-N3. Gratifyingly, compound **6** dose-dependently labeled NNMT (Figure 4C). Notably, the limit of detection is 7 pmol of NNMT (Figure 4D). These results demonstrate that NNMT methylates **6** to generate its own probe. To the best of our knowledge, this compound is the first small-molecule probe that relies on suicide-inhibition. Since **6** inhibits cellular NNMT activity, we next treated HEK293T-NNMT cells as well as cell lysates with increasing concentrations of **6** in the presence of SAM. The labeled proteins were then “clicked” to TAMRA-N3. Notably, we observed a dose-dependent increase in the labeling of NNMT by compound **6** (Figure 4E, S7). Similar results were obtained for SKOV-3 cells, which endogenously express NNMT (Figure 4F, S7). In addition to NNMT, **6** labels a small number of other proteins in both cell lines. This result can easily be explained if N-methylation is faster than enzyme inactivation and the N-methylated product can diffuse out of the active site and react with other proteins leading to off-target labeling. To confirm this possibility, we measured the partition ratio, i.e. the molar ratio of inactivator to enzyme required to completely abolish enzymatic activity. For compound **6**, this ratio is ~ 200 (Figure S8), consistent with the notion that the suicide inhibitor diffuses out of the active site and rebinds to react with C159.

In conclusion, we developed a novel suicide-inhibition strategy for NNMT, which is overexpressed in several types of cancers and many chronic diseases. This strategy utilizes 4-chloropyridine (**2**) and 4-chloronicotinamide (**3**) as NNMT substrates to generate the corresponding N-methylated products. In turn, these compounds inhibit NNMT by covalently modifying C159 which lies adjacent to the active site. Since these compounds rely on enzyme catalysis to generate the reactive species, they are mechanism-based inactivators. However, if inactivation requires release and rebinding, they could also be deemed pro-drugs.29 Experiments to distinguish these possibilities are ongoing. Building on this discovery, we also developed a suicide inhibition-based protein labeling (SIBLing) strategy using compound **6**, which efficiently labels recombinant as well as cellular NNMT. Despite their modest potency, these compounds serve as a foundation for developing more efficient NNMT inhibitors. Future efforts are focused on incorporating the 4-chloropyridine warhead into larger inhibitor scaffolds to develop suicide inhibitors with improved potency and selectivity.

**ASSOCIATED CONTENT**

**Supporting information.** The Supporting Information is available free of charge on the ACS Publications website at DOI:

Complete methods,Figures S1-S8, Tables S4, supporting references.

AUTHOR INFORMATION

Corresponding Author

\*Mailing address: Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, LRB 826, 364 Plantation Street, Worcester MA 01605. Tel.: 508-856-8492. Fax: 508-856-6215. E-mail: paul.thompson@umassmed.edu.

ORCID

Paul R. Thompson: 0000-0002-1621-3372

Notes

The authors declare the following competing financial interest(s): P.R.T. founded Padlock Therapeutics and is entitled to payments from Bristol Myers Squibb if certain milestones are met. P.R.T. is a consultant for Celgene and Disarm Therapeutics.

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**Figure Legends**

**Figure 1.** (A) NNMT reaction. (B) Compounds **1**-**6**. (C) Inactivation of NNMT by **3**. (D) Inactivation of NNMT by **1**. (E) Plot of (*k*obs) versus [I] for **1**. All experiments were performed in duplicate (n = 2).

**Figure 2.** (A) Compounds **2** and **3** are NNMT substrates. (B) Inactivation of NNMT by **2** and **3** plus SAM. Compounds (0.5 mM) were incubated with NNMT for 0.5 h and residual activity assayed. (C) Inactivation of NNMT by **2** and **3** plus SAM. Note that the errors were so small that the error bars lie within the data points. (D, E, F) The deconvoluted molecular weights for unmodified NNMT and NNMT containing an uncleaved N-formyl-methionine (NNMTa), **2**-modified NNMT, and **3**-modified NNMT. All experiments were performed in duplicate (n = 2).

**Figure 3.** (A) Active site of NNMT highlighting C159 and C165. (B) NNMT and the C159A, C165A and C159/165A mutants methylate **3**. (C) Compound **1** inhibits NNMT and the C165A mutant but not the C159A or C159/165A mutants. (D) Inactivation of NNMT, C159A and C165A by **2** and **3** plus SAM. All experiments were performed in duplicate (n = 2).

**Figure 4.** (A) Compound **6** is an NNMT substrate. Note that the errors are so small that the error bars lie within the data points. (B) Inactivation of NNMT by **6** plus SAM. Deconvoluted mass of NNMT-**6** complex (inset). (C) Concentration-dependent labeling of recombinant NNMT with **6.** F and C stand for fluorograph and Coomassie stain. (D) Limit of detection study. (E) Concentration-dependent labeling of HEK293T cell lysates (spiked with 0.5 µg of recombinant NNMT) by **6.** R stands for recombinant NNMT.(F) Concentration dependent labeling of SKOV3 cell lysates with **6.** All experiments were performed in triplicate (n = 3).