Macrocyclic Peptides as Allosteric Inhibitors of Nicotinamide N-Methyltransferase (NNMT)

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ABSTRACT: Nicotinamide N-methyltransferase (NNMT) methylates nicotinamide to form 1-methylnicotinamide using S-adenosyl-L-methionine (SAM) as the methyl donor. The complexity of the role of NNMT in healthy and disease states is slowly being elucidated and provides indication that NNMT may be an interesting therapeutic target for a variety of diseases including cancer, diabetes, and obesity. Most inhibitors of NNMT described to date are structurally related to one or both of its substrates. In search of structurally diverse NNMT inhibitors, an mRNA display screening technique was used to identify macrocyclic peptides which bind to NNMT. Several of the cyclic peptides identified in this manner show potent inhibition of NNMT with IC\textsubscript{50} values as low as 229 nM. Interestingly, substrate competition experiments reveal that these cyclic peptide inhibitors are noncompetitive with either SAM or NA indicating they may be the first allosteric inhibitors reported for NNMT.
INTRODUCTION

Nicotinamide N-methyltransferase (NNMT) is a cytosolic enzyme that catalyzes the methylation of nicotinamide (NA, vitamin B3) and a variety of other pyridines in the presence of S-adenosyl-L-methionine (SAM) to form 1-methyl nicotinamide (MNA) or the corresponding methylpyridinium ion.\(^1,2\) Recently, a number of reports have demonstrated that the role of NNMT is not limited to its involvement in xenobiotic metabolism, but rather reveal NNMT to be a master metabolic regulator in a variety of cancers\(^3\)–\(^5\). Additionally, NNMT overexpression has been described as a cancer biomarker\(^6\)–\(^10\). Aside from these roles in human cancer, NNMT is implicated in Parkinson’s Disease (PD),\(^11,12\) pulmonary arterial hypertension (PAH),\(^13\) diabetes,\(^14\) and obesity.\(^15,16\) Interestingly, these involvements can be either protecting, as in PD and PAH, or damaging as in cancer, diabetes, and obesity. To more fully understand the roles played by NNMT in healthy and disease states, specific NNMT inhibitors are needed. However, despite the increasing interest in NNMT, a limited number of NNMT inhibitors have been described to date and none have entered clinical trials\(^17\)–\(^21\).

Previous work in both our group and others has focused on the design and optimization of bisubstrate inhibitors of NNMT that incorporate structural elements of both the NA and SAM substrates. In this report, we describe the use of an entirely different strategy for the development of NNMT inhibitors. Specifically, we applied a peptide-mRNA display technology known as the random nonstandard peptide integrated discovery (RaPID) system to screen a library of more than \(10^{12}\) macrocyclic peptides binding to NNMT. This mRNA display screening technique has demonstrated promising results against a variety of protein targets with several resulting peptide macrocycles currently in clinical trials\(^24\)–\(^26\). In the present study, hits from this RaPID screen against NNMT were identified, synthesized by Fmoc solid phase peptide synthesis (SPPS), and tested for inhibition using a convenient LC-
MS-based NNMT activity assay previously developed in our group.\textsuperscript{2} Potent NNMT inhibition was found for a number of the macrocyclic peptides identified. Interestingly, this inhibition was found not to be impacted by elevated concentrations of either NA or SAM substrates suggesting that these peptides function as allosteric inhibitors, the first to be reported for NNMT.

**RESULTS AND DISCUSSION**

To identify macrocyclic peptide binders for NNMT, two parallel selections employing the RaPID system for reprogrammed mRNA display\textsuperscript{27,28} were performed using purified, \textit{N}-terminal His-tagged NNMT as the target (Fig. 1). The two selections differed only in the stereochemistry of the initiating amino acid, \textit{L}-tyrosine or \textit{D}-tyrosine, as a way of increasing the conformational space of the library. Both initiating tyrosine amino acids also carried an \textit{N}-terminal chloroacetyl moiety to give spontaneous macrocyclisation\textsuperscript{29} with a cysteine hard-coded after a stretch of 15 random amino acids. This head-to-sidechain thioether cyclized library showed exponential enrichment of target-binding sequences over the course of 6 rounds. Hits were identified by high-throughput sequencing of the output DNA from each round (see supplemental Fig. S1 in Supporting Information). Within these hits, no clear consensus sequences were visible but it was clear that hydrophobic and positively charged amino acids were overall enriched (see supplemental Fig. S2 in Supporting Information). Based on the results of the two selections performed, 17 unique peptides were selected for chemical synthesis and assessment as NNMT inhibitors (Table 1).
Figure 1. Schematic overview of the RaPID mRNA display system used to translate a random DNA library (>10^{12} library members, 17 residues), affording a large peptide library whose members were selected for binding affinity against NNMT. Selections initiated with either N-chloroacetyl-L-tyrosine or N-chloroacetyl-D-tyrosine were performed to introduce additional structural diversity in the library.

Using standard Fmoc-SPPS, the peptides were synthesized on rink amide resin as depicted in Scheme 1. The N-terminus of the linear peptide was subsequently treated with chloroacetyl chloride on the resin. After acidic cleavage and deprotection of the amino acid side chains, the peptides were cyclized in the presence of base and the macrocyclic peptides and purified using preparative HPLC. Notably, among the 17 peptides synthesized (Table 1) peptides 9 and 10 actually derive from the same sequence containing an additional Cys residue in the variable region of the sequence. For this reason in peptides 9 and 10, one of the two Cys
residues was replaced by Ala to allow for the controlled synthesis of a single macrocyclic species.

The cyclic peptides were subsequently tested for their inhibitory activity against NNMT using an LC-MS based method. The results given in Table 1 show that all 17 peptides identified from the RaPID selections demonstrate the capacity to inhibit NNMT with potent inhibition (defined as IC$_{50}$ value below 1 µM) observed for 5 of the macrocyclic peptides (see Supporting Information for full IC$_{50}$ curves). Notably, no correlation could be found between the degree of enrichment in the RaPID selection and inhibitory activity. On the contrary, the most abundant peptides from the screen, peptides 1, 9 and 10, were found to be only moderate NNMT inhibitors with IC$_{50}$ values around 5 µM. These findings suggested that the macrocyclic peptides may be interacting with NNMT at a site(s) not directly involved in the methylating activity of the enzyme.

Scheme 1. General synthesis route for the preparation of cyclic peptides identified from the mRNA display screen. The example presented contains 6 amino acids, whereas the identified peptides all contain 18 amino acids.
Table 1. Sequences, abundance and IC$_{50}$ values for selected macrocyclic peptides initiated with L-tyrosine (Y) or D-tyrosine (D). The residues in bold and the blue lines highlight the location of the thioether linkage.

| Peptide | Sequence                      | Abundance$^a$ | IC$_{50}$ (µM)$^b$ |
|---------|-------------------------------|---------------|--------------------|
| 1       | YARRILVFRDRLVVICG             | 9.5 %         | 4.964 ± 0.509      |
| 2       | YRIVIHKLYLLRIGCG              | 1.4 %         | 1.584 ± 0.111      |
| 3       | YIYFILEPGYYARVNVICG           | 1.1 %         | 1.115 ± 0.066      |
| 4       | YFIILHPRTLRALVICG             | 1.1 %         | 0.772 ± 0.054      |
| 5       | YFAITKNSRWKIIWLCG             | 0.7 %         | 1.125 ± 0.076      |
| 6       | YIFVWNNRYLVFRICG              | 0.6 %         | 0.674 ± 0.042      |
| 7       | YYVVSFGKLYLVRKCG              | 0.6 %         | 5.344 ± 0.432      |
| 8       | YTIIQLKRYLFAVHSCG             | 0.5 %         | 2.238 ± 0.211      |
| 9       | DYPKCFGIKFRDRLLLAG            | 6.6%          | 4.426 ± 0.428      |
| 10      | DYPKAFGIKFRDRLLLCG            | 5.7 %         | 6.241 ± 0.889      |
| 11      | DYTIVFRFFNLVLINC              | 5.4 %         | 1.131 ± 0.080      |
| 12      | DYTIAFILNGRLAIIVRCG           | 5.4 %         | 1.209 ± 0.113      |
| 13      | DYKQLIILSGRLILIC             | 2.6 %         | 0.241 ± 0.012      |
| 14      | DYRYLFIIAGKKEYAIIVRCG         | 1.2 %         | 7.058 ± 0.530      |
| 15      | DYTITFIRNLIDQILVFCG           | 1.1 %         | 0.437 ± 0.023      |
| 16      | DYFVFARFGNHIIVIIKACG          | 1.1 %         | 1.238 ± 0.100      |
| 17      | DYSVSVIRGRYIIGIRCG           | 0.9 %         | 0.229 ± 0.007      |

$^a$Percentage of total sequences after the sixth round of enrichment. Peptides 1-8 originate from the selection initiated with L-tyrosine and peptides 9-17 originate from the selection initiated with D-tyrosine.

$^b$Half-maximal inhibitory concentration of the compounds tested against human wild-type NNMT (full assay details provided in the supporting information). Values reported in µM are based on triplicate data of at least 10 different concentrations.

To further investigate the mode of inhibition, macrocyclic peptide inhibitors with IC$_{50}$ values below 2 µM were subsequently tested for competition with the substrate binding site(s). In this experiment, the concentration of either NA or SAM was increased 10-fold and the impact on the IC$_{50}$ value of the macrocyclic peptide inhibitor determined. As can be seen in Figure 2, none of the cyclic peptides saw a significant change in IC$_{50}$ in the presence of elevated concentrations of either of the substrates. For comparison, and as a control, two known
bisubstrate inhibitors, compounds $X^{23}$ and $Y^{18}$, previously reported to bind in the NNMT active site, were also included in the assay. In line with expectation, these bisubstrate NNMT inhibitors did show a marked increase in their IC$_{50}$ values under higher concentrations of both substrates with a more pronounced competitive effect seen at increased SAM concentrations. These findings suggest that whereas the bisubstrate inhibitors bind in the NNMT active site, the cyclic peptides instead engage with NNMT at an allosteric binding site(s) and as such are not competitive with the NA and SAM substrates.

![Figure 2. Results of the substrate competition experiment. The data is normalized per compound by setting the IC$_{50}$ under normal assay conditions at 1. Data is based on duplicate data of at least 7 different concentrations. Structures of control compounds X and Y are presented on the left.](image)

CONCLUSION

Using the RaPID mRNA display methodology, a set of macrocyclic peptides were identified with affinity for NNMT. While the hits identified from the RaPID selections did not reveal a consensus sequence, all peptides displayed a higher abundance of hydrophobic and positively charged amino acids. To investigate whether binding to NNMT resulted in the inhibition of its methylation activity, the most highly enriched cyclic peptides from both L-tyrosine and D-
tyrosine initiating libraries were synthesized using Fmoc-SPPS and subsequently evaluated for their inhibitory activity against NNMT. From the screening hits, five macrocyclic peptides showed potent inhibition with IC\textsubscript{50} values between 200 and 800 nM. Notably, none of the macrocyclic peptides showed any significant competition with the native NNMT substrates indicating that they bind at an allosteric site on the enzyme. This is the first description of allosteric inhibitors of NNMT. To further elucidate the mode of binding and potential for application of these macrocyclic peptide-based NNMT inhibitors, structural studies and cell-based assays are underway, the results of which will be reported in due course.

**EXPERIMENTAL PROCEDURES**

**General Procedures:** All reagents were purchased from Sigma Aldrich or Combi-blocks and used as received. HPLC-grade acetonitrile, peptide grade N,N-dimethylformamide (DMF) and dichloromethane (DCM) for peptide synthesis were purchased from Biosolve Chimie SARL and VWR, respectively. Ultrapure water was obtained from a Veolia Purelab flex3 water purification system. Standard Fmoc-protected amino acids and rink amide resin were purchased from P3 Biosystems.

Liquid Chromatography-Mass Spectrometry (LC-MS) was performed on a Shimadzu LC-20AD system with a Shimadzu Shim-Pack GIST-AQ C18 column (3.0 x 150 mm, 3 µm) connected to a Shimadzu 8040 triple quadrupole mass spectrometer (ESI ionization). HRMS analyses were performed on a Thermo Scientific Dionex UltiMate 3000 HPLC system with a Phenomenex Kinetex C18 column (2.1 x 150 mm, 2.6 µm) connected to a Bruker micrOTOF-Q II mass spectrometer (ESI ionization) calibrated internally with sodium formate.
Reprogrammed mRNA display protocol

Acylation of N-chloroacetyl tyrosine (both L- and D- stereochemistry in separate reactions) onto tRNA^{formylated} was carried out using amino acids synthetically activated as cyanomethyl esters and in vitro transcribed tRNA and catalyst ‘enhanced flexizyme’ as previously reported\textsuperscript{30,31} incubating for 2 hours on ice before purifying by ethanol precipitation and storing the dry pellet at -20 °C.

Two parallel selections were carried out using His tag-immobilised NNMT\textsuperscript{32} based on a previously published method,\textsuperscript{33} one with D- and one with L-tyrosine initiation. Briefly, DNA encoding 15 randomized NNK codons followed by a section encoding a CGSGSGS linker was assembled by PCR, and subsequently transcribed in vitro using T7 RNA polymerase (NEB) at 1 mL scale with 25 pmol input DNA (starting diversity \(\approx 1.5 \times 10^{13}\)) at 37 °C overnight. Following purification by PAGE, the resulting library was ligated by T4 RNA ligase at room temperature for 30 min to a short oligonucleotide terminating in puromycin before purifying by ethanol precipitation with 0.25 mg.mL\(^{-1}\) RNAse-free glycogen.

Translation of 10 pmol of this puromycin-mRNA library in vitro using the PURExpress system (combining solution A from \(\Delta\) (aa/tRNA) and solution B from \(\Delta\) RF123 kits; NEB) in a 5 \(\mu\)L reaction at 37 °C for 30 min, with methionine omitted and with added initiating acyl-tRNA to 25 \(\mu\)M, gave a cyclic peptide library with covalent mRNA tag. Following translation, the reaction mix was allowed to stand at room temperature for 12 minutes, and then EDTA was added to 16.6 mM and the reaction mix was again incubated at 37 °C for 30 min. This was then reverse-transcribed by Protoscript II reverse transcriptase (NEB) at 42 °C for 1 hour, TBS-T and BSA added from concentrated stocks to 1X and 0.1% final concentrations (respectively), and a 0.5 \(\mu\)L sample diluted to 500 \(\mu\)L. Any background peptides and proteins that bind directly to Dynabeads His-tag isolation and pulldown resin (invitrogen) were removed by three sequential incubations with free resin (5, 2.5, and 2.5 \(\mu\)L)
for 10 min at 4 °C. The supernatant following this last pre-clear step was added to immobilised NNMT saturating 0.4 µL of the same His-tag isolation and pulldown resin and incubated for 30 min at 4 °C with constant inversion. Nonspecifically-bound members of the library were removed by stringent washing on ice (3 X 20 µL TBS-T), and any surviving peptides were eluted by incubation in 50 µL RNase free water at 95 °C for 5 min and then transferring the supernatant to a new tube while hot. The first aliquot of pre-clear beads was washed and eluted in the same way as the selection containing NNMT. All samples (1 µL each of input, positive, negative) were analysed by qPCR alongside a standard curve produced by reverse transcription of the input library, and recovery was calculated as the percentage of the input found in the positive or negative selection rounds after accounting for dilution factors. The remainder of the eluted specific binders were amplified by PCR to provide a new DNA template that served as the input for the subsequent round (round 1 carried out at double scale to increase initial diversity), and selection was continued until recovery indicated enrichment above the background negative selections (Supplemental Fig. S1).

The DNA output was used for sequencing of all rounds on the Illumina MiSeq platform using a 2 X 150 bp V2 reagent kit at the Utrecht UMC sequencing facility (USEQ). The resulting sequencing output files were analysed by Python script that searches for exact matches to the T7 promoter and puromycin ligation sequences, translates the coding sequence between these, and tallies at the peptide level the abundance of each unique hit (Supplemental Fig. S2).^{34}

**Fmoc-solid-phase Peptide Synthesis (SPPS)**

**General procedure A:** All peptides were synthesized using a microwave-assisted peptide synthesizer (CEM HT12 Liberty Blue). Rink Amide AM resin (100 µmol) was swollen in 10
mL of a 1:1 mixture of DMF/DCM for 5 min, drained, and then treated with 20 vol.% piperidine (10 mL) in DMF for 65 seconds at 90°C, drained and washed with DMF (3 x 5 mL). The resin was then treated with a solution of Fmoc-Xaa-OH (0.2 mol/L, 2.5 mL, 5 eq), DIC (1 mol/L, 1 mL, 10 eq) and Oxyma (1 mol/L, 0.5 mL, 5 eq) in DMF (4 mL) at 76°C for 15 s before the temperature was increased to 90°C for an additional 110 s before being drained. The resin was then treated again with the same amount of Fmoc-Xaa-OH, DIC and Oxyma in DMF (4 mL) at 76°C for 15 s before the temperature was increased to 90°C for an additional 110 s before being drained.

**General procedure B; Manual coupling (N-terminal chloroacetyl group capping):** The resin (25 µmol) was washed with DMF (3 x 5 mL) and treated with chloroacetyl chloride (50 µmol, 4 µL, 2 eq) and DIPEA (100 µmol, 18 µL, 4 eq) in DMF (5 mL) while shaking for 1 h at room temperature. The resin was then washed with DMF (3 x 5 mL) and DCM (3 x 5 mL), respectively. The resin was dried with a nitrogen flow and used in the next step without further purification.

**General procedure C; Manual cleavage:** The peptide was cleaved from the resin using a mixture of TFA/water/TIPS/EDT (92.5:2.5:2.5:2.5) under shaking for 2 hours at room temperature. The resin was filtered over cotton and washed with TFA (2 x 0.5 mL). The crude peptide was precipitated in a mixture of MTBE/Hexane (1:1). The peptide was pelleted by centrifugation (5 min at 4500 rpm), the pellet was washed twice with MTBE/Hexane (1:1) (50 mL), centrifuged (5 min at 4500 rpm) and dried under a nitrogen flow.

**General procedure D; Manual cyclization, deprotection, and purification:** The crude peptide was dissolved in 2 mL DMSO with 10 µL DIPEA and stirred for 16 hours at room temperature to facilitate cyclization. The reaction was quenched with 10 µL TFA and the crude mixture was purified by preparative HPLC to afford the pure peptide as a white solid.
using a BESTA-Technik system with an ECOM Flash UV detector monitoring at 214 nm and 254 nm. Preparative reversed-phase HPLC was performed using a Dr. Maisch Reprosil Gold 120 C18 Prep Column (10 µm, 25 × 250 mm) using a mobile phase of water–acetonitrile gradient moving from Buffer A (5% acetonitrile, 95% water and 0.1% TFA) to 100% Buffer B (95% acetonitrile, 5% water and 0.1% TFA) over 60 minutes at a flow-rate of 12.0 mL·min⁻¹ with UV detection at 214 nm and 254 nm.

**Inhibition Studies**

Expression and purification of full-length human wild-type NNMT protein (hNNMTwt) were performed as previously described. The purity of the enzyme was confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining, and NNMT identity was confirmed using SDS-PAGE and Western blotting. Catalytic activity of the recombinant protein was evaluated with 1 unit of enzyme activity representing the formation of 1 nmol of MNA/h of incubation at 37°C. The specific activity of the batch used in the inhibitory activity assays was 15064 units/mg of protein at a protein concentration of 8.4 mg/mL. NNMT was used at a final concentration of 20 nM diluted in assay buffer (50 mM Tris buffer (pH 8.4) and 1 mM dithiothreitol). The compounds were dissolved in DMSO and diluted with water to concentrations ranging from 1 nM to 100 µM (DMSO was kept constant at 1.25% final concentration). The compounds were incubated with the enzyme for 10 min at room temperature before initiating the reaction with a mixture of NA and SAM at their $K_M$ values of 200 and 8.5 µM, respectively. The formation of MNA was measured after 40 min at room temperature. The reaction was quenched by addition of 30 µL of the sample to 70 µL of acetonitrile containing 50 nM deuteromethylated nicotinamide as internal standard. The enzymatic activity assays were performed using Multiple Reaction Monitoring (MRM) on a Shimadzu LC-20AD system with a Waters
Acquity BEH Amide HILIC column (3.0x100 mm, 1.7 µm particle size, Waters, Milford) at 65°C using water containing 300 mM formic acid and 550 mM NH₄OH (pH 9.2) at 40% v/v and acetonitrile at 60% v/v isocratically at a flowrate of 0.6 mL/min, with a runtime of 1.7 min. Calibration samples were prepared using 70 µL of internal standard d₃-MNA at 50 nM in acetonitrile and 30 µL of an aqueous solution of reference standard MNA with concentrations ranging from 1 to 1024 nM. Ratios of the sums of the MNA and d₃-MNA transitions were used to calculate concentrations of MNA. Concentrations of MNA were plotted against concentration of inhibitor and the results were subsequently normalized with the highest value in the concentration range defined as 100% inhibition. The percentage of inhibitory activity was plotted as a function of inhibitor concentration and fit using non-linear regression analysis of the sigmoidal dose-response curve generated using the normalized data and a variable slope in Graphpad Prism 8.

Substrate competition was performed under three different conditions; 1) normal conditions with both substrates at K_M values, 2) NA at 2 mM and SAM at its K_M of 8.5 µM and 3) NA at its K_M of 200 µM and SAM at 85 µM. All peptides were tested under these conditions at concentrations between 20 µM and 27 nM in duplicate using 100 nM hNNMTwt. The slightly higher concentration of enzyme compared to the initial inhibition testing was used to achieve more signal for enhanced discrimination between high and low values. The compounds were incubated with the enzyme for 10 min at room temperature before initiating the reaction with a mixture of NA and SAM. The formation of MNA was measured after 30 min at room temperature. Results were normalized to indicate the fold change in IC₅₀ value compared to the normal conditions.

**Supporting Information**

Supplementary figures, characterization data for all peptides synthesized, and IC₅₀ curves.
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