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Small molecule inhibitors of Nicotinamide N-Methyltransferase (NNMT)

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Chapter 1

Introduction: Nicotinamide *N*-methyltransferase (NNMT), an emerging therapeutic target

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Abstract

Nicotinamide *N*-methyltransferase (NNMT) methylates nicotinamide (NA) to generate 1-methyl nicotinamide. Since its discovery 70 years ago, the appreciation of the role of NNMT in human health has evolved from serving only metabolic functions to also being a driving force in diseases, including a variety of cancers. Despite the increasing evidence indicating NNMT as a viable therapeutic target, the development of cell-active inhibitors against this enzyme is lacking. In this chapter, we provide an overview of the current status of NNMT inhibitor development, relevant *in vitro* and *in vivo* studies, and a discussion of the challenges faced in the development of NNMT inhibitors.

1. Introduction

Nicotinamide *N*-methyltransferase (NNMT) (EC 2.1.1.1) is a phase II metabolizing enzyme that belongs to the family of *S*-adenosyl-L-methionine (SAM)-dependent methyltransferases.[1] In 1951, NNMT was first partially purified from rat liver by Cantoni, who subsequently discovered the structure of cofactor SAM in 1952.[2,3] In the 1990s the human and mouse NNMT genes were cloned, revealing the highly conserved nature of NNMT in mammals, with human and mouse NNMT both containing 264 amino acid residues with 92% sequence similarity and 86% sequence identity.[4–6] The closest structural homologues of NNMT are the small molecule methyltransferases indolethylamine *N*-methyltransferase (INMT) and phenylethanolamine N-methyltransferase (PNMT) having 53% and 39% sequence identity to NNMT, respectively.[4,7] NNMT catalyses the methylation of nicotinamide (NA) and a variety of other pyridine containing compounds using the methyl donor SAM to generate *S*-adenosyl-L-homocysteine (SAH) and 1-methyl nicotinamide (MNA) or the corresponding pyridinium ion (Figure 1).[8,9]

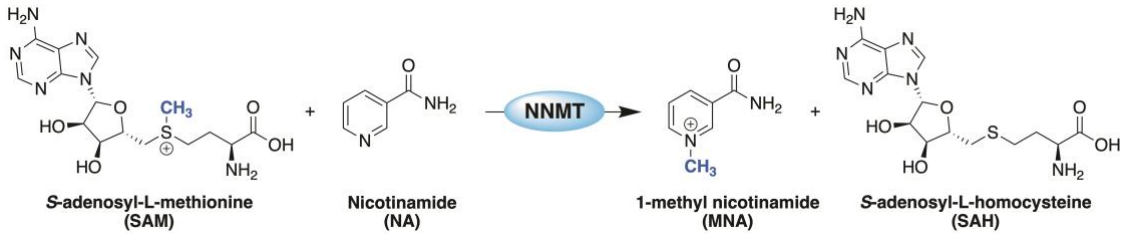

Figure 1. Nicotinamide N-methyltransferase (NNMT)-mediated methyl transfer from *S*-adenosyl-L-methionine (SAM) to nicotinamide (NA), forming 1-methylnicotinamide (MNA) and *S*-adenosyl-L-homocysteine (SAH).

The kinetics of NNMT appear to follow an ordered mechanism with SAM binding to NNMT before its pyridinyl substrate can bind. Subsequently, after the methyl transfer is completed, the methylated substrate leaves first after which SAH is released.[10] This mechanism is supported by Isothermal Titration Calorimetry data in which the binding affinity of NA could only be measured in the presence of SAH.[11] This finding suggests a significant conformational change of the enzyme upon cofactor binding, which has implications for the development of substrate competitive small molecule inhibitors of NNMT.

NNMT is found predominantly in the liver, but low levels of NNMT are also detected in most other organs.[4] It was originally thought that the primary roles of NNMT were centred around NA metabolism and detoxification of xenobiotic compounds.[12] However, more recent studies have provided evidence pointing towards a much broader function for NNMT in both healthy and disease states. NNMT is involved in the regulation of the cellular level of SAM as well as the

SAM/SAH ratio. Not only does NNMT consume SAM, but it also promotes SAM regeneration from homocysteine through interactions with betaine-homocysteine methyltransferase and methionine adenosyltransferase, both of which play key roles in the methionine cycle.[13] Furthermore, NNMT plays a critical part in NAD-dependent signalling and links the NAD^+ and methionine metabolism pathways through parallel depletion of NA and SAM.[14,15] Through these pathways, NNMT modulates energy expenditure in adipose tissue and controls glucose, cholesterol and triglyceride metabolism in hepatocytes through interaction with sirtuins.[16] Notably, in a *C. elegans* model, the activity of NNMT was found to extend lifespan by decreasing cellular SAM levels, producing a starvation signal and consequently inducing autophagy. Simultaneously, the MNA thereby formed is oxidized leading to the release of reactive oxygen species, thereby increasing stress resistance and promoting longevity.[17,18]

The elucidation of the various functions of NNMT demonstrates the complexity of the pathways in which the enzyme is involved. Not surprisingly, aberrant NNMT expression is observed in a wide range of disorders and diseases. Most pronounced in this regard is the overexpression of NNMT in a number of human cancers. Increased NNMT activity has been observed in bladder, breast, colorectal, gastric, hepatocellular, lung, oral, ovarian, pancreatic, and prostate cancer, as well as glioma, lymphoma, and insulinoma.[12,19,20] In these cancers, the overexpression of NNMT has been associated with tumour aggressiveness and shown to promote the migration, invasion, proliferation, and survival of cancer cells. At the cellular level, overexpression of NNMT facilitates epigenetic modifications by generating a metabolic methylation sink that boosts pro-tumorigenic gene products.[21] This finding was further substantiated by a recent proteomics-based study revealing NNMT to be a master metabolic regulator of cancer-associated fibroblasts (CAFs).[22] Expression of NNMT in CAFs leads to SAM depletion and decreased DNA and histone methylation levels, resulting in extensive gene expression changes in the tumour stroma, promoting cancer metastasis. A recent investigation also found that increases in MNA levels in the tumour microenvironment lead to the inhibition of T-cell functions resulting in their decreased killing capacity and increased tumour growth.[23] NNMT also interacts with oncogenic kinases, activated transducers and activators of transcription, and interleukins.[24,25] Given the absence of (cell-active) small molecule NNMT inhibitors, the role of NNMT is often studied through the use of RNA interference (siRNA or shRNA) to downregulate its expression.[24,26,27] This process occurs through inhibition of the translation of RNA to proteins in cells, resulting in lower NNMT levels, effectively mimicking inhibition of NNMT. Inhibition or down-regulation of NNMT decreases cell proliferation, reduces tumorigenicity in mice, and causes

tumour cell death via intrinsic apoptotic pathways, highlighting the potential of NNMT inhibitors as therapeutic agents.

A second disease area with increased interest in NNMT as a therapeutic target are metabolic disorders. Population studies have shown that serum MNA levels are positively correlated with obesity and diabetes.[28] In line with these findings, *Nnmt* knockdowns in mice were found to be protective against diet-induced obesity via increased energy expenditure.[29] In addition, glucose levels in *Nnmt*-knockdown mice were significantly reduced and insulin sensitivity increased.[30,31]

Aside from the clearly emerging roles in cancer and metabolic disease, links to aberrant NNMT expression have also been found in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's diseases and schizophrenia,[18,32–34] as well as functional disorders of the endothelium, such as thrombosis, high blood pressure, atherosclerosis, inflammation and pulmonary hypertension.[35]

The functions and mechanism of action of NNMT and its product MNA are not yet completely understood. The wide range of healthy and disease states in which NNMT is involved demonstrates the complexity of the role of this enzyme in human biology. To further elucidate the potential indications in which NNMT can be targeted to therapeutic benefit, potent, selective, and cell-permeable inhibitors are essential.

The first crystal structure demonstrating the active site interactions of NNMT with its substrate NA and cofactor analogue SAH was published in 2011 facilitating the development of rationally designed small molecule inhibitors of NNMT.[6] A second prerequisite for the development of inhibitors is the availability of a sensitive and specific assay for measuring NNMT activity. The first assays used for measuring NNMT activity involved the use of radiolabelled ^{3}H-methyl-SAM. In this approach NNMT activity is quantified based on the incorporation of radioactivity into the product MNA detected by scintillation counting. To avoid the use of radioactivity, a variety of alternative general methyltransferase assays as well as NNMT-specific assays have been developed, each with their own advantages and disadvantages. General methyltransferase assays that have been applied to the measurement of NNMT activity include enzyme-coupled reactions wherein the SAH by-product is subsequently detected by fluorescent or luminescent readout. Such approaches are technically straightforward and suitable for high-throughput screening (HTS). However, there are also disadvantages to this method. Firstly, the by-product SAH can be generated through degradation of SAM via either automethylation or chemical degradation pathways. Moreover, enzyme-coupled SAH detecting assays are unsuitable for use in cellular systems as the enzymes required are already present in cells, leading to interference and false positive results.[36] Another limitation of this assay is that it cannot distinguish the activity of different SAM-dependent

methyltransferases. It is therefore important that results obtained with SAH detecting are validated with an orthogonal, enzyme-specific assay.

A more specific assay for NNMT activity was developed by Sano and co-workers wherein the condensation of MNA with acetophenone results in the formation of fluorescent 2,7-naphthylpyridine analogues.[37,38] While this assay can be used in HTS, it involves significant sample workup, is an end-point assay, and, if used for inhibitor screening, also requires assessment of possible fluorescent interference by the inhibitors themselves. As an alternative, in 2016, our group reported an LCMS-based method for measuring the activity of NNMT through direct detection of MNA. The method can be used to quantify NNMT-mediated formation of MNA and a range of other positively charged, methylated pyridines with very high specificity and sensitivity.[9] While the method has a short run-time of less than 2 minutes, it is not directly suitable for HTS and is better suited for studying NNMT activity in complex mixtures and/or for validation of HTS hits. Recently, a complementary, non-coupled, real-time analytical assay for monitoring NNMT activity was reported by the Watowich group based on the fluorescent properties of 1-methylquinolinium (1-MQ).[39] This convenient method uses quinoline as an alternative substrate for NNMT instead of its primary substrate NA and relies on fluorescent detection of 1-MQ. While the method is compatible with HTS, care needs to be taken in accounting for the inherent fluorescent properties of the quinoline substrate.

In this chapter we provide a comprehensive summary of the NNMT inhibitors reported to date. The inhibition values and the analytical methods used to obtain them, are included in an overview table (Table 1) at the end of this review for ease of reference. In the subsequent sections, the following classes of NNMT inhibitors will be discussed: SAM competitive inhibitors of NNMT, nicotinamide competitive inhibitors, bisubstrate inhibitors, covalent inhibitors, and other NNMT inhibitors.

2. NNMT inhibitors

2.1 SAM Competitive Inhibitors

The by-product SAH (**1**, Figure 2), common to all SAM-dependent methyltransferases, is known as a feedback inhibitor and inhibits NNMT with an IC_{50} value of 35.3 μM.[40] SAH is only active in enzyme-based biochemical assays; it loses its activity in cellular assays were it is rapidly degraded by *S*-adenosyl-L-homocysteine hydrolase (SAHH) to adenosine and homocysteine. Another known general methyltransferase inhibitor is the natural product sinefungin (**2**, Figure 2), a SAM-mimicking methyltransferase inhibitor isolated from *Streptomyces*. Sinefungin is a moderate inhibitor of NNMT with an IC_{50} of 12.5 μM. Sinefungin has low cell membrane

permeability and exhibits severe toxicity in animal models, restricting its potential application as a therapeutic agent.[41] The moderate inhibitory activity of the SAM-mimics like SAH and sinefungin suggests that interactions in the SAM binding site alone are not sufficient for potent and selective inhibition of NNMT.

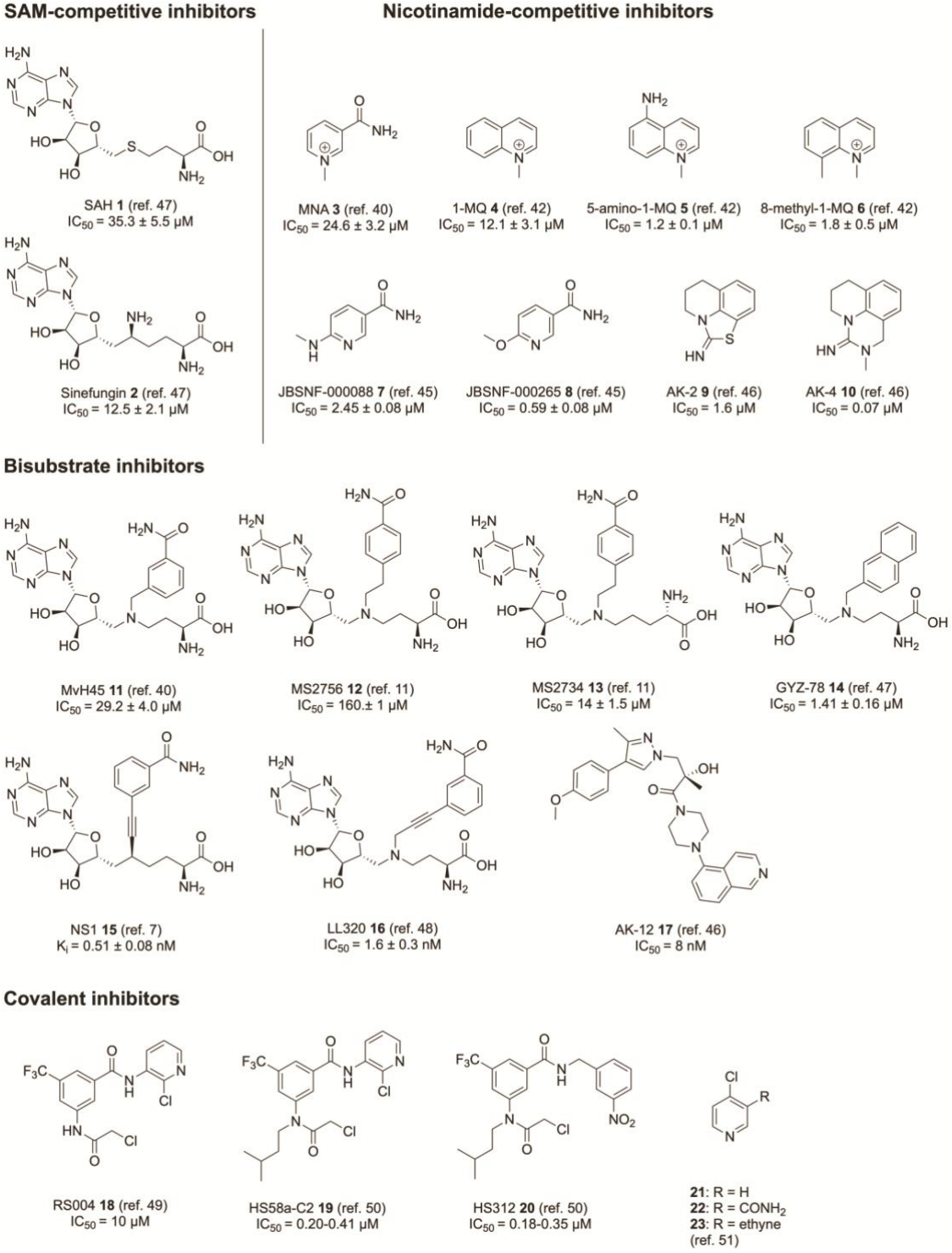

Figure 2. Overview of the chemical structures of nicotinamide N-methyltransferase (NNMT) inhibitors, including methyltransferase-specific inhibitors **1** and **2**,[47] nicotinamide-competitive inhibitors **3–10**,[40,42,45,46] bisubstrate inhibitors **11–17**,[7,11,40,46–48] and covalent inhibitors **18–23**.[49–51]

2.2 Nicotinamide Competitive Inhibitors

Inhibitors that compete with binding of the nicotinamide substrate have also been reported. As described above for the NNMT by-product SAH, the other enzymatic product, namely the methylated pyridine product MNA (**3**, Figure 2) is also a feedback inhibitor of NA methylation with comparable potency to that of SAH (IC_{50} = 24.6 μM). Similar levels of inhibition are observed for other *N*-methylated products formed from other substrate heterocycles including the *N*-methylated quinoline, 1-MQ (**4**, Figure 2) which exhibits an IC_{50} value of 12.1 μM. In a structure-activity relationship (SAR) study involving various methylated quinolines, both 5-amino-1-MQ (**5**, IC_{50} = 1.2 μM) and 8-methyl-1-MQ (**6**, IC_{50} = 1.8 μM) were shown to have improved inhibition compared with the parent compound.[42] Furthermore, in an aged mouse model, compound **5** was found to accelerate muscle regeneration, linking NNMT inhibition to functional improvements of aged skeletal muscles.[43] In addition, treatment of diet-induced obese (DIO) mice with compound **5** resulted in significantly reduced body weight and white adipose mass, decreased adipocyte size, and lowered plasma total cholesterol levels.[44]

In search of small molecule NNMT inhibitors for the treatment of metabolic disorders, an HTS screen was performed on over a million compounds.[45] The hit compound identified (JBSNF-000088, **7**, Figure 2) showed low micromolar activity against NNMT (IC_{50} = 2.4 μM), which was improved after a SAR study on this compound (JBSNF-000265, **8**, IC_{50} = 0.59 μM). Crystal structures show that compound **7** is methylated by NNMT in the nicotinamide binding site, which indicates the compounds are acting as slow turnover substrates. In high-fat DIO mice, compound **7** was able to reduce plasma levels of MNA, improve insulin sensitivity, normalize glucose tolerance, and reduce body weight.[30]

A recent publication from researchers at Sanofi reports the results of an HTS campaign in which tricyclic, nicotinamide competitive, inhibitors were identified (compound **9**, Figure 2).[46] After optimization, the most potent tricyclic inhibitor (compound **10**, Figure 2) was found to inhibit NNMT with an IC_{50} value of 0.07 μM. Co-crystallisation studies, requiring the addition of SAH, provide atomic level insight into the binding of these compounds in the nicotinamide binding pocket.

2.3 Bisubstrate Inhibitors

Based on the inhibitory activities of compounds that exclusively target either the SAM or NA binding pocket, it becomes apparent that targeting only one of these pockets may not be sufficient to achieve potent inhibition of NNMT. As an alternative, bisubstrate NNMT inhibitors have been

designed to simultaneously engage both of these binding pockets as a means of enhancing both inhibitor activity and selectivity. Our group described the first systematic approach towards the design of bisubstrate inhibitors of NNMT. From the SAR performed, it became clear that many of the functional groups present in SAM and NA are essential for binding and small alterations in the chemical structure of the bisubstrate compounds can have significant impact on their activity. The bisubstrate inhibitor MvH45 (**11**, Figure 2) linked a benzamide, mimicking NA, to an Aza-SAH moiety, mimicking SAM, resulting in moderate inhibition of NNMT (IC_{50} = 29.2 μM).[40] Building on this result, and based on the measured distance of 3.5 - 4.2 Å between the pyridinyl nitrogen atom of NA and the SAH sulphur atom as found in the NNMT crystal structure (PDB ID: 3ROD), Jin *et al*. extended the linker to the benzamide from one to two carbon atoms resulting in MS2756 (compound **12**, Figure 2) which exhibited a significantly reduced activity (IC_{50} = 160 μM). Interestingly, extension of the linker to the amino acid moiety by one carbon as in MS2734 (compound **13**, Figure 2), led to a restoration of inhibitory activity (IC_{50} = 14 μM).[11] Structural studies with compound **13** (PDB code: 6CHH) confirmed the hypothesized binding in the NNMT active site with the bisubstrate effectively recapitulating the majority of binding interactions present in the NNMT-NA-SAH ternary complex. An extensive selectivity screen on a panel of methyltransferases revealed additional activity against lysine methyltransferase DOT1L (IC_{50} = 1.3 μM) and arginine methyltransferase PRMT 7 (IC_{50} = 20 μM).

Optimization of the structural features of these bisubstrate inhibitors led us to pursue an SAR focusing on the amino acid and benzamide side-chains. From this work a naphthalene-containing compound (GYZ-78 (**14**), Figure 2) emerged with an IC_{50} of 1.4 μM. Modelling suggested that the compound benefits from additional π−π stacking interactions with several tyrosine residues in the NA binding pocket of the enzyme. No activity was found against representative members of the lysine methyltransferase (NSD2) or arginine methyltransferase (PRMT1) families and cellular data obtained for compound **14** showed a significant inhibitory effect on cell viability in HSC-2 oral cancer cells.[47] Shortly thereafter, the group of Shair found that a 2-carbon alkyne-linker provides for a superior mimicking of the orientation and distance between NA and SAM.[7] Applying an impressive multi-step stereo-controlled synthesis route, they also replaced the central nitrogen of the previous generation bisubstrate inhibitors with a carbon atom, to generate a set of highly potent NNMT inhibitors (K_i = 0.5 nM for compound NS1 **15**, Figure 2). A selectivity screen against a panel of methyltransferases, including closely related small molecule methyltransferases, revealed excellent selectivity. However, in cell-based assays, both **15** and its methyl ester prodrug only moderately decreased MNA levels in U2OS cells, most likely due to limited cell permeability. Following a similar strategy, Huang and co-workers found that the use of a three-carbon propargyl

linker to connect the central nitrogen of the first generation bisubstrate inhibitors (**11-14**) with the benzamide moiety also yielded very potent inhibitors.[48]. Among the compounds synthesized, LL320 (compound **16**, Figure 2) showed the highest activity with K_i values as low as 1.6 nM. Good selectivity was also observed against a panel of small molecule, lysine and arginine methyltransferases. As for the other SAM-based bisubstrate inhibitors of NNMT, however, both LL320 and its ethyl ester prodrug displayed poor cell permeability.

Notably, the recent HTS campaign reported by Sanofi identifying compounds **9** and **10**, also yielded compound **17** (IC_{50} = 8 nM, Figure 2) subsequently found to be a bisubstrate-like NNMT inhibitor.[46] Supported by structural insights, compound **17** represents an important step towards achieving inhibitors that less explicitly mimic the SAM and nicotinamide scaffolds. Notably, the carboxamide moiety present in nicotinamide and the amino acid side chain present in cofactor SAM are absent, while the adenosine moiety of SAM is effectively mimicked a piperazinyl-quinoline motif. While no cell-based or *in vivo* data were reported for these compounds, it will be interesting to see whether such NNMT inhibitors show improved activity in this regard.

2.4 Covalent Inhibitors

The active site of NNMT contains several non-essential cysteine residues, which can be explored as targets for covalent inhibition. The first covalent inhibitors of NNMT were identified by Cravatt and co-workers using SAH-based photoreactive probes, developed for chemical proteomic profiling of SAM-dependent methyltransferases.[49] Using these probes as a fluorescence polarization tool, an electrophilic fragment library was screened, identifying the chloroacetamide-containing covalent NNMT inhibitor RS004 (**18**, Figure 2) with a moderate IC_{50} value of 10 μM. The absence of activity against the C165A mutant of NNMT supports the interaction with a target cysteine. SAR studies on compound **18** yielded the more potent covalent NNMT inhibitors HS58a-C2 (**19**, IC_{50} = 200-410 nM, Figure 2) and HS312 (**20**, IC_{50} = 180-350 nM, Figure 2).[50] However, in cellular assays, these compounds did not show any appreciable inhibition of NNMT while interaction with other proteins was observed, contradicting the *in vitro* results. Following another approach, the Thompson group found 4-chloropyridine analogues (compounds **21-23**, Figure 2) to be substrates and inhibitors of NNMT.[51] Upon *N*-methylation of the pyridine analogue, the increased electrophilicity of the methylated pyridine promotes an aromatic nucleophilic substitution reaction by C159, a non-essential active site cysteine residue, resulting in covalent inhibition of NNMT. No IC_{50} or K_i values were given, but the K_M values of compounds **21-23** as substrates were stated as 22-44 μM. Covalency was confirmed by mass spectrometry, dialysis, and analysis of activity against C159A and/or C165A mutants of NNMT. Furthermore, in NNMT-

overexpressing HEK293T cells, the compounds showed inhibition of NNMT with EC_{50} values of 36-87 μM. In another study aimed at identifying covalent inhibitors, a library of mild electrophilic fragments was screened against a selection of cysteine-containing proteins. The screen identified several compounds that covalently labelled NNMT after incubation for 24 hours at 4°C at a concentration of 200 μM as determined by mass spectrometry.[52] However, in follow-up studies these hits did not show significant inhibition of NNMT at 200 μM.

2.5 Other NNMT Inhibitors

Another NNMT inhibitor of interest is the natural product Yuanhuadine (YD, **24**, Figure 3).[53] This compound is isolated from the flower bud of *Daphne genkwa*, which is used in traditional Chinese medicine. YD exhibits modest to potent growth inhibition of several tumour cell lines.[54–56] Lee and co-workers found that treatment of cancer cell lines with YD suppresses NNMT expression in non-small cell lung cancer (NSCLC) cells and biochemical assays indicate an IC_{50} value of 0.4 μM.[57] Docking studies suggest that YD binds in both nicotinamide and SAM binding pockets in the NNMT active site.

As another alternative source of NNMT inhibitors, our group recently applied an mRNA display technique wherein a large library of 10^{12} macrocyclic peptides was screened, resulting in a number of peptides that bind to NNMT.[58] Among the hits identified, several macrocyclic peptides were found to also potently inhibit NNMT with IC_{50} values as low as 229 nM (compound **26**, Figure 3). Interestingly, substrate competition experiments indicated that these cyclic peptide inhibitors are non-competitive with either SAM or NA, suggesting they may engage with and inhibit NNMT via an allosteric binding site. During preparation of this review article, a patent was disclosed by Eli Lilly describing a novel class of pyrimidine-5-carboxamide compounds as inhibitors of NNMT, exemplified by compound **25** in Figure 3.[59] The compound showed potent inhibition of NNMT (IC_{50} = 74 nM) in a biochemical assay as well as a dose-dependent reduction of the formation of d_4-MNA in mice dosed with d_4-nicotinamide.

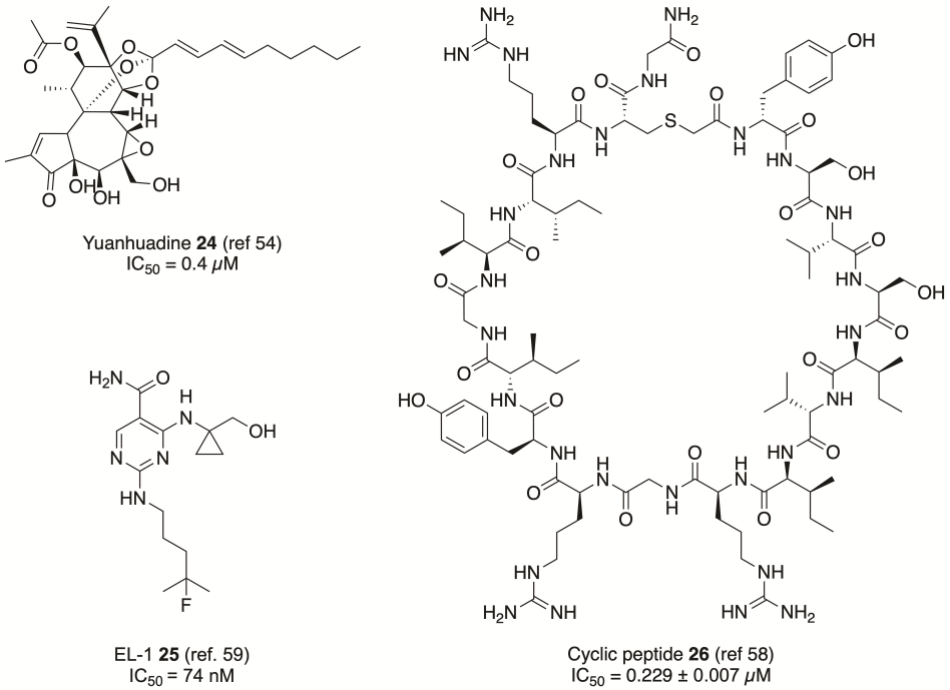

Figure 3. Chemical structures of the natural product Yuanhuadine (**24**),[54] Eli Lilly's pyrimidine 5-carboxamide compound **25**,[59] and macrocyclic peptide **26**,[58] which was found to be an allosteric inhibitor of nicotinamide N-methyltransferase (NNMT).

3. Conclusion

In this review, we present an overview of the current state of NNMT inhibitor development and highlight their advantages and drawbacks. While the search for effective NNMT inhibitors is still in its infancy, substantial progress has already been made in terms of potency and selectivity of small molecule inhibitors of NNMT. That said, the limited cellular and *in vivo* activity of these compounds speaks to the need to develop more drug-like inhibitors. The clinical importance of NNMT in a variety of diseases, including cancer and metabolic disorders, support NNMT as a viable therapeutic target. However, major challenges remain in developing NNMT inhibitors for clinical application. The SAR studies performed on bisubstrate inhibitors of NNMT reveal the importance of highly polar functional groups, including the adenosine and amino acid moieties of the SAM-mimetics. However, while these features are critical for activity, they are also detrimental to cell permeability. In order to establish the therapeutic viability of NNMT inhibition, the current set of NNMT inhibitors available needs to be expanded to provide more cell-permeable probe molecules. With such inhibitors in hand, it will be possible to more precisely assess the beneficial and detrimental effects, both acute and chronic, of NNMT inhibition in cellular systems and *in vivo* models.

Table 1. Overview of NNMT inhibitors with IC_{50} or K_i values, analytical methods used and results from *in vitro* and *in vivo* studies

No.	Name	IC_{50}	Analytical method	*In vitro/in vivo* results	Refs
1	SAH	35.3 ± 5.5 μM	LC-MS	N/A	47
2	Sinefungin	12.5 ± 2.1 μM	LC-MS	N/A	47
3	MNA	24.6 ± 3.2 μM	LC-MS	N/A	40
4	1-MQ	12.1 ± 3.1 μM	HPLC	No data	42
5	5-amino-1-MQ	1.2 ± 0.1 μM	HPLC	Accelerated muscle regeneration in aged mice; reduced body weight and white adipose tissue in diet-induced obese mice; treatment of human CAFs increased histone methylation and did not affect cell viability. Decreased tumour burden in mouse model of ovarian cancer metastasis, reduced tumour cell proliferation, and increased stromal H3K27 trimethylation	42–44
6	8-Methyl-1-MQ	1.8 ± 0.5 μM	HPLC	No data	42
7	JBSNF-000088	2.4 ± 0.1 μM	2,7-Naphthyridine fluorescence	Reduced body weight, improved insulin sensitivity, and restored glucose tolerance in mice with diet-induced obesity	30,45
8	JBSNF-000265	0.6 ± 0.1 μM	2,7-Naphthyridine fluorescence	No data	45
9	AK-2	1.6 μM	2,7-Naphthyridine fluorescence	No data	46
10	AK-4	0.07 μM	2,7-Naphthyridine fluorescence	No data	46
11	MvH45	29.2 ± 4.0 μM	LC-MS	No data	40
12	MS2756	160 ± 1 μM	SAHH-coupled fluorescence	K_D (ITC) of 42.8 ± 6.3 μM	11
13	MS2734	14 ± 1.5 μM	SAHH-coupled fluorescence	K_D (ITC) of 2.7 ± 0.2 μM	11
14	GYZ-78	1.4 ± 0.2 μM	LC-MS	K_D (ITC) of 5.6 ± 0.4 μM; reduced cell viability of HSC-2 oral cancer cell line	47
15	NS1	0.5 ± 0.1 nM(Ki)	Quinoline fluorescence	Moderately decreased MNA levels in U2OS osteosarcoma cells	7
16	LL320	1.6 ± 0.1 nM (Ki)	SAHH-coupled fluorescence	No Data	48
17	AK-12	0.008 μM	2,7-Naphthyridine fluorescence	No data	46
18	RS004	10.0 μM	ABPP probe/FP	No data	49
19	HS58A-C2	410/200 nM	ABPP probe/LC-MS	Good NNMT inhibition in lysates of human renal cell	50
20	HS312	350/180 nM	ABPP probe/LC-MS	carcinoma line 786O; however, despite cell permeability, no cellular NNMT inhibition reported	50
21–23	4-Chloropyridine analogs	N/A	MTase Glo/quinoline fluorescence	K_M values of 22–44 μM; inhibition of NNMT in HEK293T cells with EC_{50} values of 36–87 μM	51
24	Yuanhuadine	0.4 μM	SAHH-coupled fluorescence	Suppression of NNMT expression in NSCLC cells	57
25	EL-1	74 nM	LC-MS	Dose-dependent reduction of MNA in mice	59
26	Cyclic peptide	0.229 ± 0.007 μM	LC-MS	Noncompetitive inhibition indicated allosteric binding	58

4. Thesis Outline

The theme of this thesis is the development of small molecule inhibitors of NNMT with the aim of increasing their potency and selectivity. To this end, structural modifications were performed on a bisubstrate NNMT inhibitor previously identified in our group.[40] These investigations are described in **chapter 2**. A diverse library of inhibitors was prepared to probe the different regions of the enzyme's active site, revealing that incorporation of a naphthalene moiety intended to bind the hydrophobic nicotinamide binding pocket via π-π stacking interactions significantly increases the activity of the bisubstrate-like NNMT inhibitors. These findings were further supported by ITC binding assays as well as modeling studies. The most active NNMT inhibitor identified in the present study demonstrated a dose-dependent inhibitory effect on the cell proliferation of an HSC-2 oral cancer cell line.

To further increase the potency of NNMT inhibition, we next developed a series of compounds that depart from some of the conserved structural features found in the first generation bisubstrate inhibitors, specifically by introducing alternative electron-deficient aromatic groups to mimic the

nicotinamide moiety (**Chapter 3**). In addition, the identification of an optimal trans-alkene linker differs from the previously reported alkyl and alkynyl linkers used to connect the substrate and cofactor mimics in these inhibitors. The most potent NNMT inhibitor identified in our study exhibited an IC_{50} value of 3.7 nM placing it among the most active NNMT inhibitors reported to date. Complementary analytical techniques, modelling studies, and cell-based assays provide insight into these inhibitors' binding mode, affinity, and selectivity.

While our best compound showed potent NNMT inhibition in biochemical assay, it was lacking good cellular activity. In order to translate the observed potent affinity into strong cellular activity, a prodrug strategy was developed, which is described in **chapter 4**. The prodrug strategy focused on the temporary protection of the amine and carboxylic acid moieties of the highly polar amino acid side chain present in potent bisubstrate inhibitor. The modification of the carboxylic acid into a range of esters in the absence or presence of a trimethyllock (TML) protecting group at the amine group yielded a range of prodrugs. Based on good stability in buffers and the confirmed esterase-dependent conversion to the parent compound, the isopropyl ester was selected as the preferred acid prodrug. The isopropyl ester and isopropyl ester-TML prodrugs demonstrated improved cell permeability and translated into cellular activity.

In **chapter 5**, the most potent bisubstrate inhibitor described in chapter 3 was used as a scaffold to incorporate covalent warheads targeting cysteine and serine residues in the NNMT active site. While non-essential for catalytic activity, these residues are involved in substrate binding within the NNMT active site. Using a series of acrylamide and chloroacetamide containing compounds to target cysteine residues C159 and C165, as well as a series of sulfonyl fluoride and boronic acid containing compounds targeting serine residues S201 and S213, several compounds were identified with potent inhibitory activity against NNMT. Establishing the covalent nature of their interactions is part of an ongoing investigation.

Chapter 6 provides a summary of the results obtained in thesis chapters 1-5.

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