HHS Public Access

Author manuscript

*ACS Chem Biol*. Author manuscript; available in PMC 2021 July 17.

Published in final edited form as:

*ACS Chem Biol*. 2020 July 17; 15(7): 1747–1758. doi:10.1021/acschembio.0c00314.

# NMT as a glycine and lysine myristoyltransferase in cancer, immunity, and infections

**Tatsiana Kosciuk**[1,3], **Hening Lin**[1,2]

[1]Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853, USA.

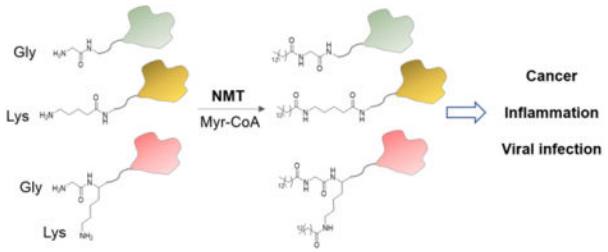
[2]Howard Hughes Medical Institute; Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853, USA.

[3]Department of Molecular Biology and Genetics Cornell University, Ithaca, NY 14853, USA

## Abstract

Protein myristoylation, the addition of a 14-carbon saturated acyl group, is an abundant modification implicated in biological events as diverse as development, immunity, oncogenesis, and infections. N-myristoyltransferase (NMT) is the enzyme that catalyzes this modification. Many elegant studies have established the rules guiding the catalysis including substrate amino acid sequence requirement with the indispensable N-terminal glycine, and a co-translational mode of action. Recent advances in technology such as the development of fatty acid analogs, small molecule inhibitors, and new proteomic strategies, allowed a deeper insight into the NMT activity and function. Here we focus on discussing recent work demonstrating NMT as also a lysine myristoyltransferase, the enzyme's regulation by a previously unnoticed solvent channel, and mechanism of NMT regulation by protein-protein interactions. We also summarize recent findings of NMT's role in cancer, immunity and infections, and the advances in pharmacological targeting of myristoylation. Our analyses highlight opportunities for further understanding and discoveries.

## Graphical Abstract




## Introduction

N-myristoyltransferase (NMT) is a ubiquitous eukaryotic enzyme that for decades has been known to have a single role – myristylation of the amino group of the N-terminal glycine on proteins. This function is of high specificity guided by several factors such as substrate

Corresponding author: Hening Lin, hl379@cornell.edu.

sequence requirements, protein interactions, and expression levels. N-myristoylation is the addition of a 14-carbon chain to the alpha amine (Nα) of the N-terminal glycine on proteins, exposed after the initiator methionine is cleaved by methionine aminopeptidase during translation, or after caspase cleavage during proteolytic events such as apoptosis. This modification often regulates protein-membrane or protein partner binding and is thought to be irreversible although there is some evidence of an ATP-dependent demyristoylase in the cytoplasmic fraction of the brain synaptosomes.[1] Recent findings implicate N-myristoylation in protein stability, where free N-terminal glycine is recognized by E3 ubiquitin ligases leading to degradation of unmyristoylated proteins.[2] NMT is essential for the survival of many eukaryotic organisms such as *S. cerevisiae*[3] and pathogens *C. albicans*, *T. brucei*,[4] *C. neoformans*[5], and many viruses utilize host NMT for replication and infectivity,[6] which made the enzyme an attractive therapeutic target. Furthermore, NMT knockout causes embryonic lethality in mice and *Drosophila*, pointing to its role in develepment.[7, 8] Here we discuss the recent advances in understanding the NMT catalysis, especially its activity on lysine residues, and the enzyme's potential for therapeutic use in cancer, immunity and infectious diseases, including viral infections. We note the areas that warrant further investigation such as reevaluation of eukaryotic and viral proteomes for lysine substrates of NMT and provide a brief update on the progress in the development of NMT-selective small molecule inhibitors.

## Structure and regulation of NMT catalysis

Several studies have elucidated that the catalytic mechanism of NMT follows an ordered bi-bi reaction where myristoyl-CoA binds to the enzyme in a bent fashion resembling a "question mark", which causes a conformational change allowing the peptide substrate to bind (Figure 1A). The interaction of the thioester carbonyl with the backbone amides of the enzyme (Phe 247 and Leu 248 in human NMT1) forms an oxy-anion hole activating the carbonyl for the nucleophilic attack by the Gly2 amine of the peptide substrate.[9, 10] This reaction was thought to occur only with the N-terminal glycine, not any other amino acids because the absence of a side chain allows free rotation of the N-terminal amine necessary for the nucleophilic attack on the carbonyl carbon of the thioester bond of myristoyl-CoA.[11] Later we will discuss recent studies that elegantly demonstrated the activity of NMT on lysine. Recently, a detailed reaction mechanism revealed that the C-terminus carboxylate of Gln496 acts as an indirect catalytic base initiating the water-mediated deprotonation of the N-terminus amino group. The carboxylate is located at the end of a 22 Å long previously unnoticed solvent channel that is thought to be important for the deprotonation.[10] After catalysis, CoA is released followed by the myristoylated product.[12] In addition to the N-terminal Gly, the peptide substrate specificity is dictated by other residues near the N-terminus. Although not an absolute requirement, NMT often prefers substrates with the sequence GXXXSK.[13–16] Ser 6 interacts with a small hydrophilic pocket explaining the enzyme's preference for this position.[13, 14, 16] Lys 7 of the peptide substrate allows a tight binding and occupies a distinct negatively charged pocket with three aspartate residues that stabilize the substrate binding via a salt bridge between the Asp carboxylate and the epsilon amino group (Nε) of the lysine.[14, 16] The sequence recognition is rather mosaic suggesting that NMT can accommodate various substrates and that other factors such as interacting

partners might regulate substrate specificity in cells.[15] For instance, the known NMT substrate ARF1 has the N-terminal sequence GNIFAN, which does not conform to the NMT preference. This example emphasizes the need to keep an open mind while examining potential NMT substrates. Furthermore, there are variations in sequence preference across species,[14, 16, 17] suggesting the need for species-specific predictive tools of N-terminal myristoylation, which can be of use for developing NMT-targeted therapies.

The exceptional preference of NMT for myristoyl-CoA remains puzzling given that in cells myristic acid comprises less than 1% of total fatty acids compared to about 20 % for palmitic acid.[18] Structural work suggests that the length of the NMT hydrophobic pocket is best suited for a myristoyl chain,[9] which partially explains this selectivity. Yet under some conditions, NMT can efficiently utilize other acyl-CoAs. For instance, the introduction of a triple bond at C6 of the acyl chain of palmitoyl-CoA results in a strong enhancement of the activity of *S. cerevisiae* Nmt, which is thought to result from inducing a bend in palmitoyl-CoA where it would naturally occur in myristoyl-CoA bound to NMT.[19] It was also shown that on the peptide GARASVLS-$NH_2$ derived from the HIV Gag protein the myristoyl-peptide formation was progressively decreased as the acyl-CoA chain was shortened with nearly no acylation upon shortening to 7 carbons.[20] Whether these trends of acyl-CoA utilization efficiency depend on the peptide substrate in vitro and in cells is unclear. Yet some reports suggest a similar binding affinity for myristoyl-CoA and palmitoyl-CoA[21, 22] and explain that NMT is “protected” from other acyl-CoAs by the acyl-CoA binding protein ACBD6.[22–24] The ACBD6 binding to NMT via its ankyrin-repeat motif (ANK) allosterically activates the enzyme for myristoyl-CoA acceptance independently from the acyl-CoA binding domain of ACBD6. The ANK domain alone is able to stimulate NMT and it produces the same effect when attached via the linker domain to ACBD1 protein, which otherwise does not regulate NMT.[24] At the same time, myristoyl-CoA bound to ACBD6 can be transferred to NMT. When NMT is introduced to myristoyl-CoA bound ACBD6, the formation of myristoyl-peptide is observed suggesting that ACBD6 hands myristoyl-CoA over to NMT; and this process is potentiated by ACBD6 phosphorylation at Ser106 and Ser108.[23] Of note is that ACBD6 binds to NMT2 about 10-fold better than to NMT1 potentially acting as a regulator of the differences between the two NMTs.[22] While it is clear that ACBD6 acts as an activator of NMT, the underlying mechanism warrants clarification on whether and how it guards the acyl-CoA specificity or whether it allosterically promotes peptide substrate binding. NMT does not seem to efficiently utilize acyl-CoAs with shorter chains such as the far more abundant acetyl-CoA, likely because the binding affinities of these short-chain acyl-CoA are too low.

There is also evidence for substrate-specific regulation of NMT activity. The cellular senescence-inhibited gene (CSIG) was shown to enhance the interaction between NMT1 and the serine/threonine protein phosphatase PPM1A promoting PPM1A myristoylation, which enhances its phosphatase activity on SMAD2 to inhibit TFG-β signaling.[25] Other interactions might regulate NMT localization. While NMT is thought to be largely cytosolic, its interactions with calnexin[26] and ribosomes[27] are thought to keep some of the enzyme at the endoplasmic reticulum.

In vitro, a truncation of 28 amino acids from the N-terminus of NMT1 catalytic domain resulted in a ~3 fold increase in the enzyme activity suggesting that additional post-translational modifications can further modulate the enzyme function.[28] During apoptosis, proteolytic cleavage regulates NMT localization. Both human NMT1 and NMT2 are cleaved in a time-dependent manner upon induction of apoptosis with Staurosporine and anti-Fas antibody in Hela and Jurkat T cells.[29, 30] NMT1 is cleaved by Caspases-3 and −8 at Asp81, while NMT2 is cleaved at Asp 25 by caspase-3. This leads to the re-localization of NMT1 from the membrane to the cytosol, while the opposite is observed for NMT2.[30] The truncated NMTs appear to remain the same activity level, but are thought to recognize substrates that can act either in a pro-survival or pro-apoptosis manner. At the same time proteolysis generates new substrates of NMT.[29] For example, a pro-apoptotic protein BID is cleaved by caspase 8, which exposes an N-terminal glycine on a 15 kDa fragment that gets myristoylated by NMT.[31] This targets the cleaved BID to the outer mitochondrial membrane to promote cytochrome c release and subsequent cell death. The discovery of this sophisticated molecular switch regulating apoptosis opened an avenue for understanding the mechanism of posttranslational myristoylation by NMT.

Human NMT1 and NMT2 are 77% identical at the amino acid sequence with the greatest divergence at their N-termini. In basal conditions they appear to have largely overlapping substrates, so their differential processing during proteolytic stress might result in more divergent substrate profiles, yet this warrants more investigation. Interestingly, NMT2 knockdown in SKOV-3 cells induced apoptosis to a greater extent than NMT1 knockdown hinting that the two human NMT enzymes might have different functions in regulating cell survival.[32] The catalytic domains of NMT1 and NMT2 are nearly identical and the most divergence comes from the N-terminus. There is no structure reported for the N-terminus of NMT1 or NMT2, which hinders the understanding of this region. The two NMTs have nonoverlapping roles as NMT2 is typically unable to compensate for NMT1 depletion. Further investigation into the potential regulation of this region by metabolite binding, protein or nucleic acid interactions and post-translational modification might shed light on the need for the two NMT enzymes in higher eukaryotes.

## NMT is the first mammalian lysine fatty acyl transferase

It was recently established that human NMT1 and NMT2 also act as mammalian lysine fatty acyl transferases. While lysine fatty acylation has been known to occur on mammalian proteins for nearly two decades[33], the enzymes catalyzing such lysine modifications remained unknown. The amino group of the N-terminal glycine and that of the lysine side chain are chemically similar displaying similar lengths and steric properties where both are free to rotate, hinting that both could react in the active site of NMT. The modification of a Lys3 residue would be similar to the modification of a Gly2 (Figure 2). In vitro and in cells NMT can myristoylate lysine residues positioned near the N-terminus with decreased catalytic ability as the lysine moves away from the N-terminus.[34] The overall catalytic mechanism appears similar to that of the N-terminal glycine, yet the structural data suggest that the solvent channel may not facilitate the reaction on lysine as the C-terminus catalytic base interacts directly with the Nε in contrast to the indirect interaction through a water molecule with the Nα of the N-terminal glycine.[10] The first cellular lysine myristoylation

substrate is the small GTPase ADP-ribosylation factor 6 (ARF6), which has a Lys3 following the N-terminal Gly2. After the reaction at the N-terminus, the Gly-myristoyl might move into the solvent channel positioning Lys3 into the catalytic center. These studies expand the repertoire of the NMT substrates: it can act on glycine, lysine, or both when located at the N-terminus (Figure 1B).

Interestingly, unlike other GNAT family enzymes, NMT contains two GNAT domains thought to result from gene duplication[35] with the solvent channel in the second domain. This channel is lined with hydrophobic residues and is of a similar length to the canonical myristoyl pocket allowing the speculation that while the second domain lost its catalytic function, it might have retained its ability to hold a myristoyl group, which can explain the di-myristoylation ability of NMT. The second pocket, therefore, may serve as both the facilitator of the deprotonation event during Gly myristylation and the binding pocket for the Gly-myristoyl moiety during di-myristoylation. While the NMT sequence preference for lysine myristoylation is similar to that of glycine, in vitro and cellular studies suggest that the reaction on lysine might be regulated by the 3D structure of the substrate protein. Interestingly, in cells, NMT2 appears a better di-myristoyltransferase than NMT1, which again suggests an additional level of cellular regulation of this function.

Unlike glycine myristoylation, lysine myristoylation is reversible. SIRT2 is the eraser of ARF6 lysine myristoylation which is important for its localization and activation. Interestingly, it appears that SIRT2 prefers the GDP-bound form of ARF6 as the substrate, while NMT preferentially acts on ARF6-GTP. This substrate specificity allows the lysine myristoylation-demyristoylation cycle to connect to and drive the GTPase cycle (Figure 3). The newly identified reversible lysine myristoylation-demyristoylation cycle opens an avenue for future studies to further characterize the new function of NMT, identify additional substrates, and understand its other functional consequences.

## Functional effects of N-myristoylation

N-myristoylation most often serves as a protein membrane targeting signals such as that in the ARF, Src, and G protein families. The hydrophobic myristoyl chain can insert into the lipid bilayer anchoring proteins to membranes where they get activated and perform their roles. The G2A mutants of N-terminally myristoylated proteins are typically inactive. To date, no erasers of N-terminal glycine myristoylation have been identified, but the membrane-binding cycle is known to be regulated by other switch mechanisms. $Ca^{2+}$-dependent myristoyl switches have been described for several proteins: among them are hippocalcin,[36] recoverin[37], and visin-like protein 3.[38] The ARF proteins, like ARF1, use nucleotide binding to turn the myristoyl switch on and off. In the GDP bound state, the N-terminal helix along with its myristoyl chain is sequestered into a hydrophobic pocket, but the exchange to GTP facilitated by GEFs causes a conformation change that extrudes the myristoylated N-terminal helix allowing its insertion into membranes. Upon GTP hydrolysis accelerated by GTPase activating proteins (GAPs), the myristoyl moiety is again sequestered releasing the ARF protein from the membrane (Figure 3).[39, 40] A myristoyl binding site was recently identified in the SH3 domain of c-Src. In the presence of lipid membranes, the myristoyl group is released and inserted into the lipid layer allowing c-Src activation and

transforming ability.[41] How c-Src switches between membrane-bound and cytosolic forms is an area of active research. In contrast to c-Src, the elegant study by Hantschel et al. demonstrated that the N-terminal myristoylation of another tyrosine kinase c-Abl does not regulate its membrane binding and is inhibitory to the enzyme's kinase activity.[42] The G2A mutation abrogates myristoylation leading to a dramatic increase in phosphotyrosine levels and kinase activity of c-Abl. Surprisingly, the mutation does not affect c-Abl localization as demonstrated by subcellular fractionation and macroscopy studies. This is a rare case of a myristoylation control of an enzyme activity that is independent of membrane binding.

A second membrane targeting signal such as a basic patch or palmitoylation of a nearby cysteine is often necessary for an efficient plasma membrane binding. Such mechanisms are utilized by the Src and G proteins where cysteine palmitoylation is catalyzed by one of the 23 human DHHC enzymes.[43, 44] The lysine myristoylation on ARF6 discussed above also represents a second membrane targeting signal that is regulated by the enzyme SIRT2. ARFs 1–5 reside in the Golgi and regulate the ER-Golgi transport while ARF6 is found at the plasma membrane and endosomes and, unlike other ARFs tends to remain membrane-bound during its GTPase cycle (Figure 3).[45] The second acylation on lysine allows efficient membrane anchoring of ARF6 and membrane retention even when it is inactive.[34] Further studies are needed to understand how widespread this mode of regulation is in the mammalian proteome.

## N-myristoylation promotes cancer progression

Targeting human NMT has recently emerged as a therapeutic strategy to treat cancer. Colon, gallbladder, and brain tumors have been shown to have elevated levels of NMT suggesting the reliance of cancer cells on protein myristoylation.[46–48] The elevation of NMT2 in osteosarcoma might be contributing to the chemoresistance of this disease.[49] Similarly, a down-regulation of miR-181c that among other genes targets NMT2 is found in imatinib-resistant chronic myeloid leukemia.[50] This suggests that NMT2 levels can be used as a predictor of response to chemotherapeutics. Interestingly, an NMT inhibitor PCLX-001, which is also called DDD86481[51] (Figure 4), is effective against NMT2 deficient blood cancers and is being advanced to clinical trials.[52] The fact that it works well in NMT2-deficient blood cancers could be related to the chemoresistance effect of NMT2. Alternatively, it could be that lower levels of NMT2 make the cells more easily to be inhibited by PCLX-001.

There could be multiple mechanisms for the anticancer effects of NMT inhibition. In breast cancer, including triple-negative breast cancer, the knockdown of NMT1 suppresses tumor initiation, proliferation, and invasion by promoting oxidative and ER stress, which in turn activates autophagy via the JNK pathway.[53] Quantitative proteomics revealed that the ER stress followed by cell cycle arrest and apoptosis was also induced in breast and colon cancer cell models upon small molecule-mediated NMT inhibition.[54] The inhibition of the cell cycle was also observed in prostate cancer upon NMT knockdown or inhibition with a myristoyl-CoA analog B13. This cell cycle arrest led to the suppression of malignant growth and invasion, which occurred via the inhibition of Src myristoylation and subsequent downregulation of its oncogenic signaling.[55] In MCF7 estrogen receptor-positive breast

cancer cells, mTOR inhibition with rapamycin leads to NMT1 upregulation.[56] NMT1 promotes lung cancer cell viability by supporting mitophagy. This occurs via the AMPKβ myristoylation that is required for the AMPK recruitment to the mitochondria where it physically associates with ATG16 and ATG5–12 and recruits VPS34 and ATG16 during mitochondrial damage. NMT1 mediated targeting to mitochondria is sufficient to initiate mitophagy and to promote cancer cell survival.[57] Thus, NMT plays an important role in multiple cancers. However, given the NMT regulation of different substrate proteins in both normal cells and cancer cells, anti-cancer targeting of NMT would benefit from selective inhibition of NMT in cancer cells over normal cells.

## NMT in immune responses

It is becoming more evident that NMT acts as a guardian of the immune response by regulating myelopoiesis, lymphopoiesis, innate immune response, and the immunological synapse.[58] NMT1 is essential to monocytic differentiation during which the NMT activity is modulated by the expression levels of the enzyme and its inhibitor protein HSC70. NMT1 deficient mouse embryonic fibroblasts are strongly impaired in differentiating into macrophages.[59] N-myristoylation also appears essential for thymocyte development and function. In a mouse model with a lineage-specific T cell deficiency in NMT1 and NMT2, T cell development and activation are severely impaired suggesting that N-myristoylation positively regulates immune response. Unlike the NMT1 thymocyte mutant mice, the NMT2 mutants demonstrate a largely wild type phenotype, but the double mutation strongly enhances the inhibition of T cell development and reduction in T cell numbers in the thymus, blood, lymph nodes, and spleen. The NMT deficiency leads to increased apoptosis during all stages of T cell development, decreased Erk phosphorylation, and mislocalization of myristoylated proteins.[60] Interestingly, in rheumatoid arthritis (RA) NMT appears to play an anti-inflammatory role. T cells from RA patients have low NMT1 levels which suppress the myristoylation-dependent AMPK lysosomal translocation and activation. This leads to hyperactivation of mTORC1 signaling in RA T cells promoting their differentiation into pro-inflammatory Th1 and Th17 cells aggravating RA. In a humanized mouse model where synovitis was induced by transferring human peripheral blood mononuclear cells and ingrafting human synovial tissue, NMT1 overexpression had a strong anti-inflammatory effect.[61] The inefficient mitophagy due to impaired AMPK function[57] may reconcile this observation in RA with the necessity for NMT activity during T cell development.[60] Mitochondrial dysfunction is the hallmark of RA and is thought to promote inflammation,[62, 63] which might be aggravated by the impaired clearance of damaged mitochondria as a result of suppressed AMPK myristoylation in RA.[64]

T cell maturation depends on the Notch1 signaling pathway.[65, 66] This might occur through the myristoylated Neutralized like-1 E3 ubiquitin ligase that facilitates the turnover and trafficking of Jagged, the Notch receptor ligand.[67, 68] In developed T cells, myristoylation of LCK governs an intricate trafficking mechanism of the kinase and is necessary for the LCK localization to the T cell immune synapse, the interface between the target cell and the T cell. The myristoyl group anchors LCK to membranes and acts as a signal for UNC119A binding that allows LCK extraction from the membranes and delivery to the synapse where it is released with the help of ARL3 and ARL13B.[69] LCK phosphorylates immunoreceptor

tyrosine-based activation motifs (ITAMs) that regulate signaling cascades essential for T cell activation. Myristoylated Fyn and c-Src orchestrate centrosome and actin-dependent movements during immune synapse.[58, 70] These reports point to a multifaceted and context-dependent role of NMT in immune response which warrants further work to aid informed therapeutic interventions.

## Parasitic NMT as a therapeutic target in malaria and sleeping sickness

Eukaryotic parasites have their own single NMT, which is being explored as a potential therapeutic target in infectious diseases like malaria and sleeping sickness. Malaria is caused by the infections with the species of the genus *Plasmodium* such as *Plasmodium falciparum* and leads to nearly a million deaths worldwide annually. The low vaccination efficacy and development of resistance to available therapies call for new therapeutic strategies. In 2014, over 30 NMT substrates were identified in *P. falciparum* using chemical proteomics. These proteins are involved in a range of functions such as protein trafficking, migration, development, and signaling pathways, suggesting that NMT is necessary for the parasite viability. This study identified a promising small molecule displaying selectivity towards PfNMT over hNMT and the ability to inhibit parasite viability and invasion of red blood cells. Excitingly, this agent caused a strong reduction in the rodent malaria parasite *P. berghei* in mice without acute toxicity to the animals.[71] A more recent high-throughput screening identified 23 chemical classes of inhibitors that were selected for *Plasmodium* NMT over the human NMT enzymes, but further lead optimization is needed for in vivo testing.[72] To address a potential resistance of the parasite to NMT inhibition, another study identified the PfNMT G386E mutant that conferred resistance to the NMT inhibitor IMP-1002. DDD85646 could overcome this resistance suggesting that a combinatorial approach might be of use when targeting NMT in this disease.[73]

Sleeping sickness is another deadly infection caused by the *Trypanosoma brucei* parasites. Similar to *P. falciparum*, *T. brucei* has its own single NMT with more than 60 predicted substrates, some of which have been experimentally validated, and is being explored as a target against sleeping sickness. Administration of the NMT inhibitor DDD86546 cures trypanosomiasis in mice potentially through disruption of the endocytic pathway by inhibiting TbARF1 myristoylation.[74] A recent chemical proteomics study in the clinically relevant bloodstream form of the parasite identified 53 high confidence and 10 medium confidence NMT substrate hits, many of which overlapped with the predicted group. Among these substrates were phosphatases, ARF GTPases, calpain-like proteins, and several uncharacterized proteins.[75] *T. brucei* infection proceeds in two stages where it dwells in the bloodstream during the first stage and infects the nervous system in the second stage. This indicates the need for drugs able to cross the blood-brain barrier and a few promising leads were recently found.[76, 77] While it is clear that NMT is a promising therapeutic target in malaria and sleeping sickness, the understanding of the underlying mechanisms is lacking. Further identification of specific NMT-regulated substrates and pathways essential to parasite viability might facilitate the pathogen-specific therapeutic intervention.

## Viral utilization of the host NMT

NMT has been widely explored as a therapeutic target for viral infections because many viruses use host NMT for increased pathogenesis (Table 1). Some of the known myristoylated viral proteins are the VP4 of poliovirus, hepatitis B virus pre-S1 protein and Gag and Nef of simian and human immunodeficiency viruses (SIV and HIV).[78] Myristoylation of Nef and Gag are essential to HIV type-1 replication and virulence. Nef myristoylation induces endocytosis of CD4 on the surface of T cells, which prevents superinfections detrimental to cell survival,[79] and interestingly it is preferentially myristoylated by NMT2.[80] Myristoylation of Gag is necessary for its membrane association followed by the assembly and budding of new viral particles.[81] NMT1 enhances the replication of HIV type-1 by increasing the expression of viral RNA.[82, 83]

Most picornaviruses such as poliovirus and coxsackievirus utilize host NMT for capsid protein myristoylation. The capsid protein VP0 is cleaved to VP2 and VP4 and shares the same myristoylated N-terminus with VP4. Genetic knockout of NMT1 or NMT2 in HAP1 cells demonstrated that only NMT1 is necessary for viral replication. Pharmacological inhibition of NMT with DDD85646 led to a strong reduction of myristoylation of VP0 and suppression of particle infectivity. Surprisingly, electron microscopy studies revealed that these particles were identical to the control particles and did not display a defect in cell attachment suggesting that lack of myristoylation might cause a defect in the transfer of viral RNA into the host cell.[84] However, the myristoylation of VP0 of rhinoviruses, the cause for respiratory diseases such as common cold, is necessary for the assembly of the viral capsid to support viral replication.[85] Interestingly, parechoviruses and kobuviruses that do not undergo VP0 cleavage are not affected by NMT inhibition suggesting that VP0 processing confers reliance on NMT.[84]

Dengue virus, the cause of dangerous dengue fever, appears to rely on NMT as well. NMT is upregulated during the infection of dendritic cells and it interacts with the viral envelope protein, while NMT gene silencing significantly suppresses dengue virus replication.[86] Thus far myristoylation of the Dengue viral proteins has only been predicted but lacks experimental proof, which is also true for many other viruses.[6] Coronaviruses such as those that caused SARS and COVID-19 pandemics are thought to lack myristoylation because they do not contain the preferred NMT recognition sequence, however, this has no experimental evidence.[78] Given the recent finding of the NMT activity towards lysine and the fluidity of NMT substrate recognition preference, it might be of use to reevaluate viral proteins for potential lysine substrate sites of NMT. For instance, the N-terminal sequence of SARS-CoV nsp4 protein is KIVSTCFK, and nsp4 of SARS-CoV2 and some bat viruses contain similar motifs with the N-terminal lysine, which raises the possibility that they are modified by NMT.

Viral protein myristoylation might be important in generating an immune response. X-ray crystallography analyses revealed that MHC class I proteins Mamu-B*05104 and Mamu-B*098 contain a large hydrophobic pocket that can bind a myristoylated glycine of the peptides C14-Gly-Gly-Ala-Ile and C14-Gly-Gly-Ala-Ile-Ser, respectively, which are derived from the viral Nef protein. Gly2 and Ala3 or Ile4 are exposed for the recognition by the

cytotoxic T lymphocytes to initiate their activation.[87] While myristoylation of viral proteins has been known for decades, its inhibition as a therapeutic strategy is still at the stage of exploration. Given the importance of NMT to the function of normal cells, its inhibition might be toxic, therefore small molecule inhibitors with increased specificity for viral protein myristylation are needed. This could be aided by the identification of regulatory proteins of NMT. For instance, it is tempting to speculate that targeting ACBD6-NMT2 interaction could preferentially inhibit myristoylation of Nef, a better substrate of NMT2 than NMT1, given the preference of ACBD6 for NMT2 over NMT1.

## Pharmacological targeting of NMT

Because of the roles of NMT in cancer and parasitic and viral infections, inhibition of N-myristoylation has been explored as an attractive therapeutic strategy against these diseases. 2-hydroxymyristic acid, D-NMAPPD (B13), or Tris-DBA palladium, IMP-366 (DDD85646), PCLX-001(DDD86481) and IMP-1088 are most commonly used pan-N-myristoylation inhibitors (Figure 4). The high structural similarity between NMT1 and NMT2 poses a challenge for the design of NMT1/NMT2-selective inhibitors and there have been no reports of such selective small molecules so far. A recent comparison of the potency and selectivity of the widely used NMT inhibitors revealed that 2-hydroxymyristic acid, D-NMAPPD, and Tris-DBA palladium are poor inhibitors of NMT. D-NMAPPD and Tris-DBA palladium cause off-target mediated cell cytotoxicity. In the same study IMP-366 (DDD85646) and IMP-1088 appeared as highly specific and potent inhibitors of NMT1 and NMT2 with nanomolar $IC_{50}$ values.[111]

Small molecule NMT inhibition has shown therapeutic potential in viral and parasitic infections and cancer. 2-Hydroxymyristic acid was shown to suppress replication of enterovirus 71,[112] while the recently discovered IMP-1088 had a therapeutic potential against rhinovirus by inhibiting capsid protein myristoylation.[85] DDD85646 is the most widely used NMT inhibitor that can suppress picornavirus replication via the host NMT inhibition,[84] as well as malaria and sleeping sickness parasites by inhibiting their NMTs.[73–75] In addition, DDD85646 suppressed breast and colon cancer cell growth by inducing ER stress and consequent cell cycle arrest and apoptosis.[54] PCLX-001 (DDD86481) is being advanced to clinical trials for treating hematologic cancers by Pacylex, which represents the most promising NMT inhibitor in cancer treatment. An anticancer effect was also achieved with D-NMAPPD, a myristoyl-CoA analog that has been reported to suppress prostate cancer progression by inhibiting Src myristoylation,[55] and Tris-DBA palladium that was effective against melanoma.[113] Further understanding of the differences in structure and regulation between human and pathogenic NMTs and between human NMT1 and NMT2 could aid the development of small molecules with increased selectivity and decreased toxicity.

## Conclusions and outstanding questions

In recent years much has been learned about the catalytic mechanism and physiological functions of NMT. This knowledge opened several exciting and important research avenues. Given the nearly identical catalytic domains of the two human NMT enzymes, the necessity

for both remains elusive and is likely hidden in the divergence of their N-termini that could potentially differ in protein, metabolite, or nucleic acid binding and posttranslational modifications. This area remains underexplored perhaps in part due to the unstructured nature of these regions. Furthermore, additional work is needed to identify NMT regulators and to understand the NMT selectivity for myristoyl-CoA and its mechanism of activation by ACBD6.

NMT has recently emerged as the first mammalian lysine myristoyltransferase with so far a single protein substrate, ARF6. Interestingly, in cells, NMT2 appears as a more potent lysine transferase than NMT1 due to an unknown level of regulation. It is of interest to further characterize the differences in the two activities of NMT, identify new lysine substrates while including viral proteins in evaluation, and determine the physiological implications of the lysine modification. Exploring conditions of proteolysis could aid the identification of new substrates followed by finding lysine myristoylation erasers such as sirtuins or HDACs. In cancer, there is a need for more mechanistic understandings of how NMT controls the oxidative and ER stress and whether NMT regulates cancer stemness. The role of NMT in immunity appears context-dependent, which is important to consider in evaluating NMT as a modulatory node for inflammation. Having this knowledge could aid in the development of highly selective NMT inhibitors with therapeutic potential.

## Acknowledgments

The work was supported by the NSF Graduate Research Fellowship Program and SUNY Diversity fellowship as well as NIH/NIDDK grant R01DK107868.

## Keywords

***Myristoylation***
addition of a 14-carbon saturated acyl chain to proteins

***N-myristoyltransferase or NMT***
the enzyme that catalyzes N-terminal myristoylation

***Lysine fatty acylation***
addition of long-chain fatty acyl groups to lysine residues

***Virus***
an infective particle that replicates inside a host cell

***Solvent channel***
structural feature of some enzymes thought to regulate deprotonation

***Membrane anchor***
typically a lipid modification that facilitates protein membrane binding

***Inhibitor***
small molecule that suppresses a function of a protein

***Cancer***

a disease caused by uncontrolled proliferation of malignant cells in the body

***Parasite***

disease-causing organism of protozoa, helminths, or arthropods

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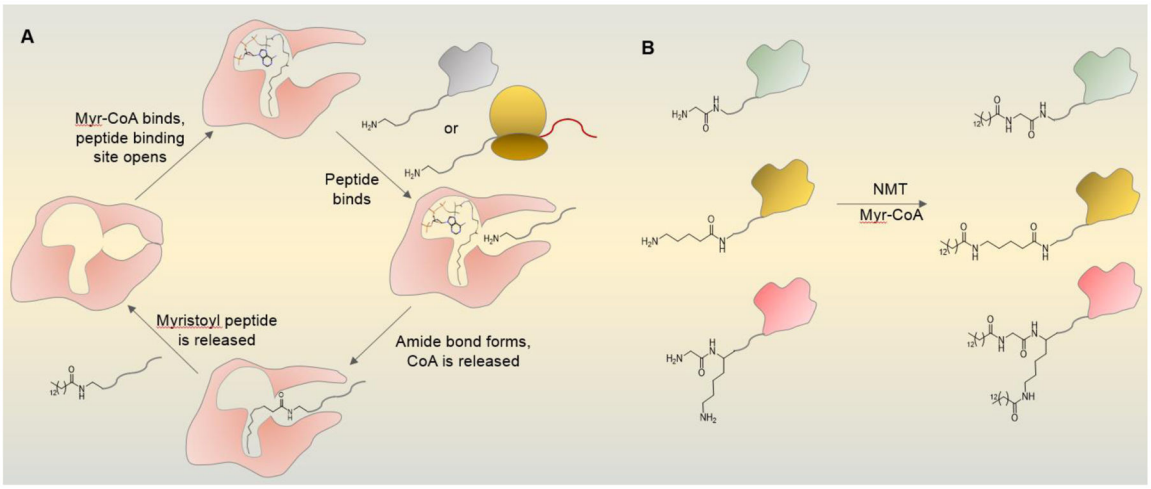


**Figure 1.**

a) Mechanism of NMT-catalyzed myristoylation. Myristoyl-CoA binds to NMT in a question mark-like fashion. This causes a conformational change that opens the peptide-binding site. Peptide substrate of a folded protein or nascent peptide with a sterically unhindered N-terminal amino group binds to NMT allowing amide bond formation between the amino group and the myristoyl group and release of CoA. The modified peptide exits the active site and the enzyme is ready for the next catalytic cycle. b) NMT can myristoylate the amino groups of N-terminal glycine, lysine, or both.

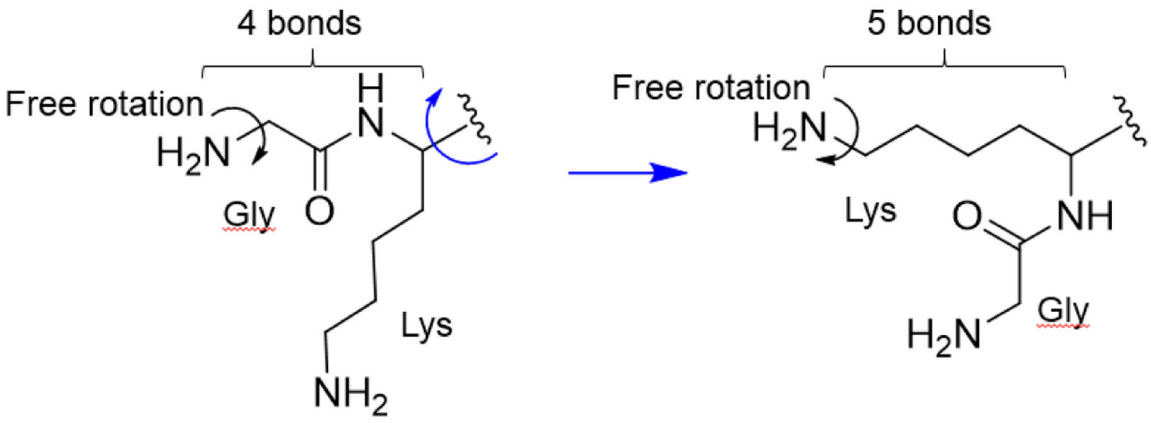


**Figure 2.**

Similarities between the N-terminal glycine and lysine. In both cases, the amino groups are free to rotate and are a similar distance away from the Cα of lysine.

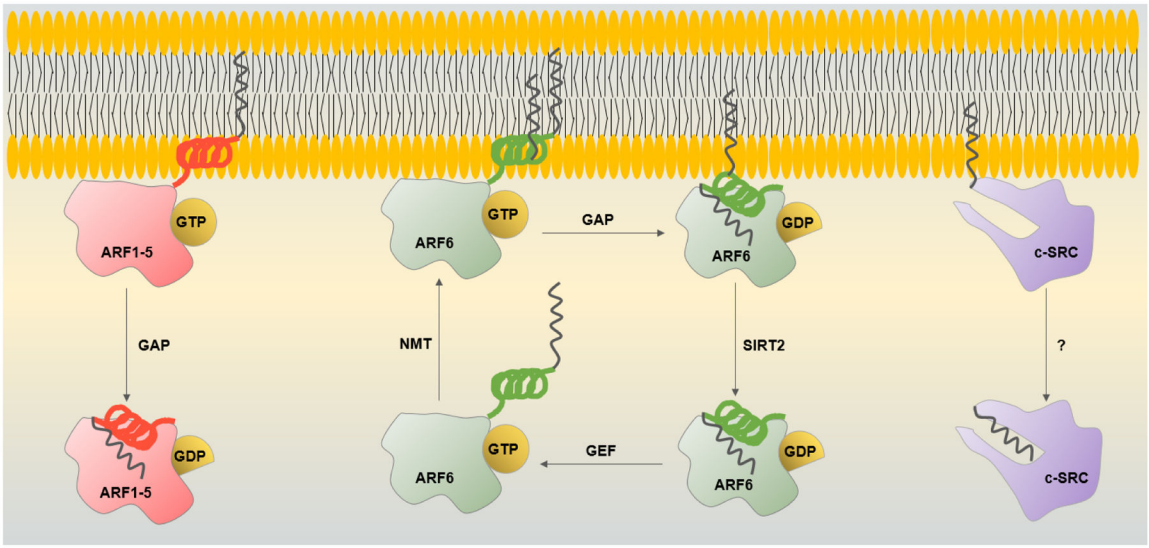


**Figure 3.**

Myristoyl switches of ARF GTPases and c-Src. ARF1–5 are membrane-bound in the GTP-bound state via their amphipathic helix with one myristoyl group. The GTP hydrolysis facilitated by GAP causes a conformational change that sequesters the myristoylated helix releasing ARF1–5 from membranes. ARF6 has an additional myristoyl chain on lysine 3 that helps to retain ARF6-GDP on the plasma membrane and endomembranes. SIRT2 removes lysine myristoylation allowing ARF6-GDP to be activated by GEF. NMT myristoylates lysine 3 of ARF6-GTP, promoting its plasma membrane localization and completing the cycle. c-Src contains a hydrophobic pocket in its SH3 domain, that sequesters the myristoyl chain, but what causes the switch is unclear.

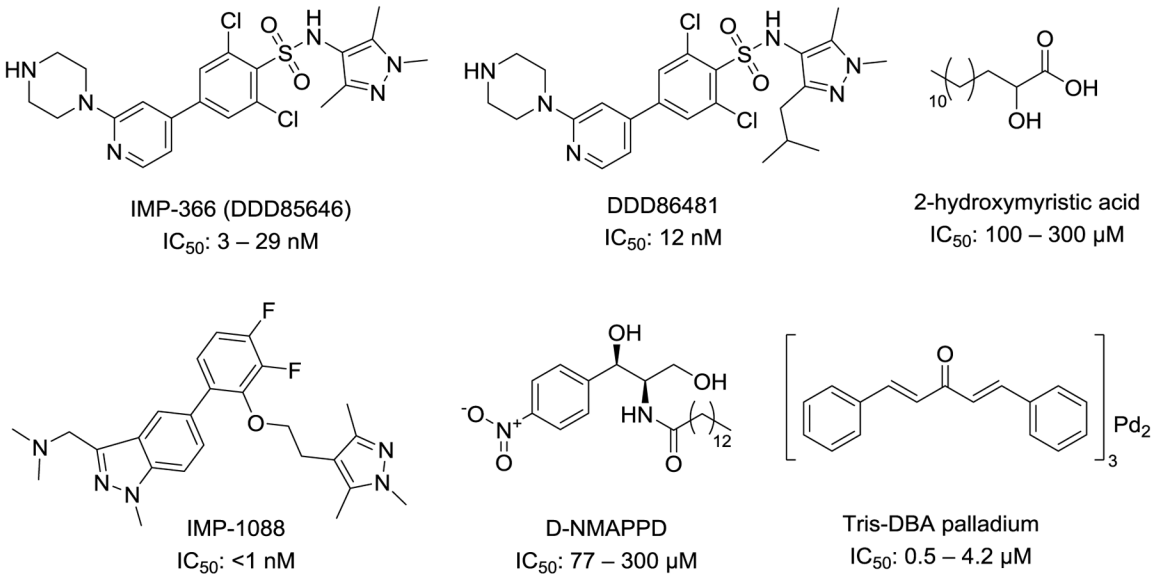


**Figure 4:**
Structures and $IC_{50}$ of small molecule NMT inhibitors.

**Table 1:**

Myristoylated viral proteins. The table was adapted from Maurer-Stroh and Frank Eisenhaber, Trends in Microbiology, 2004.[6]

| Protein | Virus | Function | N-terminal sequence | Ref. |
|---|---|---|---|---|
| Nef | Lentiviruses (HIV, SIV) | Membrane targeting to downregulate immune cell surface molecules | GGKWSKSSI | 88 |
| Gag | Retroviruses, Poxviruses (HIV) | Binds plasma membrane to initiate viral particle assembly | | |
| ❖ P17matrix protein | Lentiviruses (HIV, SIV, FIV) | | GARASVLSG | 89 |
| ❖ P15 matrix protein | Gammaretroviruses (e.g. murine and feline leukemia viruses, rat and avian sarcoma virus, avian spleen necrosis virus, baboon and porcine endogenous retroviruses, reticuloendotheliosis virus), human endogenous retrovirus S71, Poxviridae (Fowl pox) | | GQTITTPLS | 90 |
| ❖ P10 matrix protein | Betaretroviruses (mouse mammary tumor virus, Mason-Pfizer monkey virus, simian retrovirus 2, ovine pulmonary adenocarcinoma virus), wooly monkey sarcoma virus, human endogenous retrovirus K, hamster intracisternal A-particle and python endogenous retrovirus | | GVSGSKGQK | 91 |
| ❖ P19 protein of nucleocapsid inner layer | Deltaretroviruses (human/simian T-cell lymphotropic virus, bovine leukemia virus) | | GQIFSRSAS | 92 |
| VP4 coat protein | Picornaviridae [rhino-, cardio-, aphtho-, entero- (echo-, coxsackie-, polio-) viruses] | Virion assembly | GAQVSTQKS | 93 |
| Large surface antigen | Hepadnaviridae (hepatitis B virus) | Essential for viral infectivity but not assembly | GQNLSTSNP | 94 |
| L1R envelope protein | Poxviridae [Chordopoxvirinae (e.g. variola, vaccinia)and Enteropoxvirinae], Asfiviridae, Iridoviridae (lymphocysti-, rana-, iridovirus), Ascoviridae (Ascovirus) | Membrane targeting and virion assembly | GAAASIQTT | 95, 96 |
| v-src | Rous sarcoma virus | Membrane binding necessary for oncogenic transformation | GSSKSKPKD | 97, 98 |
| A16L late protein | Poxviridae [Chordopoxvirinae (e.g. variola, vaccinia) and Enteropoxvirinae] | Protein associates with viral, not cellular, structures | GAAVTLNRI | 99, 100 |
| VP2 coat protein | Polyomaviruses | Viral assembly and infectivity | GAALALLGD | 101, 102 |
| G9R | Poxviridae [Chordopoxvirinae (e.g. variola, vaccinia)] | Protein associates with viral, not cellular, structures | GGGVSVELP | 99 |
| UL11 | Alphaherpesvirinae (simplex-, varicello-, Marek's disease-like and infectious laryngotracheitis-like viruses) | Association with lipid rafts | GLSFSGARP | 103, 104 |
| UL99, U71, BBLF1 | Betaherpesvirinae (roseolo-, cytomegalo-, muromegalovirus), Gammaherpesvirinae (e.g. lymphocryptovirus), Alphaherpesvirinae | Association with ERGIC necessary for virion assembly | GAELCKRIC GAKCCKPVS GALWSLCRR | 105 |
| Major outer capsid protein μ1 | Ortho- and aquareoviruses | Cleavage of μ1 to μ1C | GNASSIVQT | 106 |
| E7R | Orthopoxviruses | Soluble protein | GTAATIQTP | 99 |
| Polyprotein pp220 | Asfivirus | Potential membrane-anchor regulating core assembly | GNRGSSTSS | 107, 108 |

| Protein | Virus | Function | N-terminal sequence | Ref. |
|---|---|---|---|---|
| Membrane fusion protein p15 | Orthoreovirus | Required for membrane fusion activity | GQRHSIVQP | 109, 110 |