N-Myristoyltransferase inhibition as a tool for antileishmanial drug discovery: Use in high-throughput, *de novo*, and piggyback strategies for drug development

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Leishmaniasis is a disease caused by the *Leishmania* genus of protozoan parasites, and spread by the phlebotomine genus of sandfly. The disease is caused by at least 20 different species of *Leishmania* parasites, and manifests itself in three forms: cutaneous, mucocutaneous, and most severely, visceral leishmaniasis. Current chemotherapeutic treatments for leishmaniasis are limited by parasite resistance to existing drugs, highly toxic side effects, and high cost of treatment. As a neglected tropical disease, there has been relatively little research toward new drugs, despite a large need, comprising 1.3m new cases and 20,000-50,000 deaths annually. Fortunately, although there is a wide variety in species causing the disease, visceral leishmaniasis is largely caused by a single species of parasite, *Leishmania donovani*, greatly simplifying the search for drugs against the most dangerous form of the disease. The whole genome of the species has been sequenced, and many crystal structures of key proteins have been elucidated. Specifically, *N*-myristoyltransferase (NMT) has emerged as a promising enzyme for target-based antileishmanial drug development, with a wide array of tools available for any level of drug discovery: *in silico* modeling and docking, enzyme specific assays for protozoan and human isoforms, high throughput *in vitro* assays against both the parasite itself and infected host cells, and animal models of the disease. However, with *L. donovani* NMT as a newer target, research has yet to utilize all of these individual elements synergistically with themselves, or

with related studies in different parasite species. Combined analysis of these methods can yield a more efficient search for antileishmanials, regardless of screening type.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF EQUATIONS

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LIST OF ABBREVIATIONS AND SYMBOLS

ADMET	absorption, distribution, metabolism, excretion, and toxicity
BALB/c	albino, laboratory-bred strain of house mouse
C57BL/6	common, inbred mouse strain
*Ca*NMT	*Candida albicans* NMT
ClogP	calculated LogP, P= [ocatanol]/[water]
DNDi	Drugs for Negelcted Disease Initiative
GSK	GlaxoSmithKline
HAT	human African trypanosomaisis
HCS	high-content screening
HIV	human immunodeficiency virus
*Hs1*NMT	*Homo sapiens* NMT1
*Hs2*NMT	*Homo sapiens* NMT2
HTS	high-throughput screening
IC_{50}	half-maximal inhibitory concentration
IV	intravenous
K_i	inhibitory constant
K_m	Michaelis-Menton constant
*Ld*NMT	*Leishmania donovani* NMT

LE	ligand efficiency
LELP	ligand efficiency dependency lipophilicity
*Lm*NMT	*Leishmania major* NMT
NMT	*N*-Myristoyltransferase
NTD	neglected tropical disease
PDB	Protein Data Bank
PKDL	Post kala-azar dermal leishmaniasis
*Pv*NMT	*Plasmodium vivax* NMT
QSAR	qualitative structure-activity relationship
SAR	structure-activity relationship
*Sc*NMT	*Saccharomyces cerevisiae* NMT
SPA	scintillation proximity assay
SPPS	solid-phase peptide synthesis
*Tb*NMT	*Trypanosoma brucei* NMT
THP-1	Human-acute monocytic leukemia cell line
VL	visceral leishmaniasis

1.0 LEISHMANIASIS: DISEASE OVERVIEW AND NEED FOR TREATMENT

Leishmaniasis is a disease caused by the *Leishmania* genus of protozoan parasites and spread by the phlebotomine genus of sandfly. The disease is endemic to tropical and sub-tropical countries, predominantly affecting the developing world.[1] Major risk factors of the disease include negative socioeconomic conditions, malnutrition, population mobility and environmental changes, all conditions frequently encountered in the tropics.

1.1.1 Disease burden

Leishmaniasis affects approximately 12 million people worldwide, with approximately 2 million new cases and 20-50 thousand deaths annually. After malaria, it is the second highest parasitic killer. The disease manifests itself in a variety of forms: cutaneous, mucocutaneous, and visceral. Cutaneous results in open sores that eventually heal on their own within a few months to a year and a half, but scarring can occur. Mucocutaneous can affect the skin and mucous membranes (nose, mouth, throat), yielding sores that typically heal, with scarring and potential tissue damage. Visceral leishmaniasis (VL, also known as 'kala-azar'), the most serious form, is caused by parasite migration to internal organs and is fatal if left untreated.[2] Even with treatment, visceral leishmaniasis can result in organ damage. Further complications can also arise from leishmaniasis infection, including post kala-azar dermal leishmaniasis (PKDL) in which the

parasite infection recurs affecting the skin and co-infection in patients with HIV. Leishmaniasis is recognized as a neglected tropical disease (NTD).[3-4] While the definition criteria vary among different health organizations, NTDs are generally endemic to low-income areas of the tropics, and that lack adequate available treatments.

Current treatments for leishmaniasis have improved in the last decade with the introduction of Miltefosine as the first orally-available therapy, but existing treatments are limited by factors of cost, oral availability, toxicity, and parasite resistance.[5] Unfortunately, parasite resistance is not only a problem of older treatments such as pentavalent antimonials, but instances of resistance have also been shown for more recently developed treatments as well.[6-9] The Drugs for Neglected Disease Initiative (DNDi) is non-profit organization devoted to developing treatments for neglected diseases, and includes a large variety of industry, governmental, and academic partners, with a focus on collaborative projects. Based on DNDi efforts, the treatment options for leishmaniasis are improving, but slowly.[10] Currently, the most advanced therapies focuses on combination of existing drugs.[11] Only one novel chemotheraputic, sitamiquine, is currently in development through GSK/Walter Reed Army Institute that is currently in phase II clinical trials. Other treatments under development are oral and topical formulations of existing drug amphotericin B, which has previously only available via IV therapy and is highly toxic. Also, fexinidazole, a human African trypanosomaisis inhibitor, is beginning phase II trials as a treatment for *L. donovani* infection as well.[12]

Palatnik-de-Sousa and Kedzierski have both proposed that the parasite's biology may provide a route to vaccination and there is one candidate vaccine currently in Phase I trials. [13-15] Nevertheless, until a range of treatments is approved for use, a need persists for the pre-clinical development of antileishmanials that meet all the desired criteria for safety, efficacy, and cost.

1.1.2 Complications to developing treatments

Leishmania parasites are transferred to mammalian hosts via the bite of infected female phebotomine sandflies, depicted in Figure 1.[16] Within the sandfly midgut, the parasites exist as motile metacyclic promastigotes, eventually migrating to the stomodeal valve. When the fly bites a host, promastigote transmission occurs, however, the vector is not yet infective. Promastigotes invade mammalian macrophages where they are phagocytized into amastigotes, the spread of which by infection and replication within host cells affects the surrounding tissue. If transmitted back to the sandfly, the amastigotes revert back to procyclic promastigotes in the absence of host macrophage cells. Undergoing cell division, parasites are considered metacyclic promastigotes, thereby restarting the cycle.

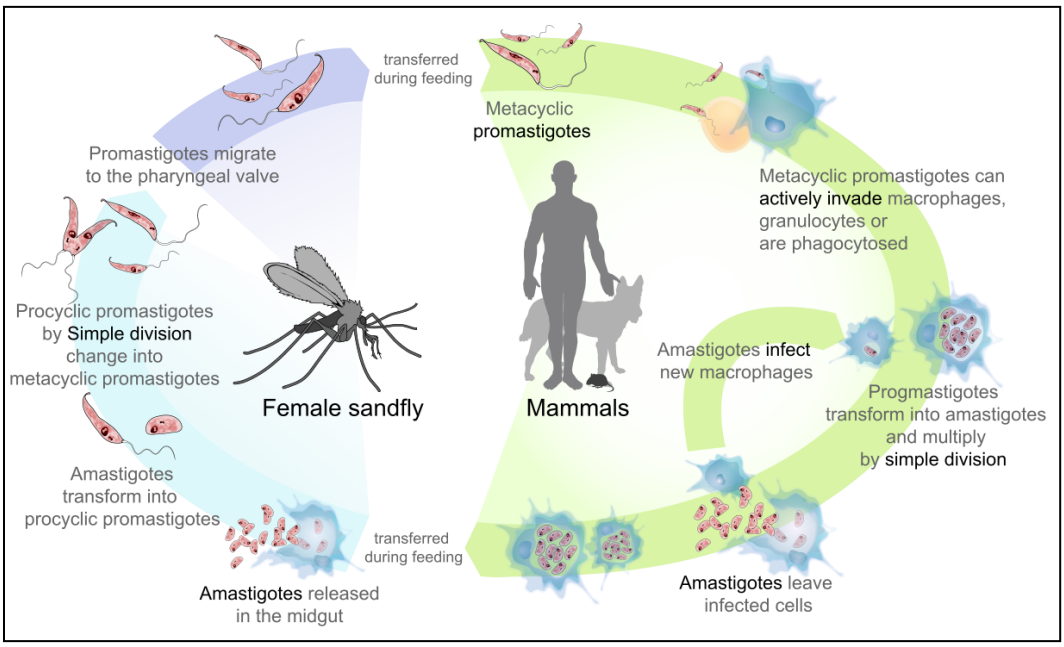


Figure 1. *Leishmania* parasite life cycle.
Image used from public domain.

In addition to the multiple parasite forms, the number of Leishmania species that cause the disease complicates disease progression, expression, and treatment development; over 20 species of parasite have been shown to cause the differing forms of the disease. This biological diversity accounts for the phenotypic differences in disease manifestation. Most species are associated with a specific form of illness, but occasionally, individual species themselves will cause different manifestations.[17] Fortunately, a wide array of genomic and proteomic data is known for *leishmania* parasites, including completely sequenced genomes of several species, the characterization of many proteins necessary for parasite function, and the solution of several crystal structures of relevant proteins.[18-20] The wealth of data regarding protein structure and function offers some mechanistic insight into known NMT inhibitors that are effective across multiple *Leishmania* species.[21] However, the number and variety of species also illustrates the complexity of generating broad-spectrum antileishmanial theraputics.

Drug development for leishmaniasis has been assisted by related research on other trypanosomatid diseases that include *Trypanosoma brucei, (*human African trypanomaisis, "sleeping sickness"), and *T. cruzi* (South American trypanosomiasis, "Chagas diease").[22] Similar drugs/targets have also been identified in certain fungal and bacterial infections, and in the *Plasmodium* genus, responsible for malaria.[23] These species share biological similarities with *Leishmania* spp. and offer a large dataset related to development of antileishmanials, including crystal structures of related target proteins, and examples of cross-species inhibition by single compounds.[24] Again, the additional information helps the understanding of existing cross-species drug efficacy, but only further convolutes an effort for development of novel broad-spectrum antitrypanosomal drugs. It is worth noting that, despite certain macromolecular similarities

between species, species pathology ultimately affects drug development as well. Considerations for HAT drug development must account for blood-brain-barrier penetration to treat the infected host. Under similar preliminary screening conditions, this would be an added complication for HAT inhibitors, but is absent from *Leishmania* inhibitor design considerations.

The number and complexity of leishmaniasis manifestations renders the disease an incredibly difficult candidate for rational drug design. Although a potent treatment against all forms of the disease may be unlikely, visceral leishmaniasis infection is both a major contributor to the global mortality and morbidity, and surprisingly well suited for rational drug design. Of the global leishmaniasis disease burden, visceral leishmaniasis accounts for approximately 200,000-400,000 new infections, and 20,000-40,000 deaths per year.[25-26]

1.1.3 Visceral leishmanias as focus for treatment

Treatment of visceral leishmaniasis is aided by the high degree of similarity between causative species. Visceral leishmaniasis is largely cited as caused by the species *L. donovani*, and occasionally, by *L. infantum.* In fact, four individual *Leishmania* species have been proposed as causes for VL: *L. donovani, L. infantum, L. chagasi,* and *L. archibaldi*.[27] Despite the general classification as separate species, the distinction largely arises from geographic origins of each sample. The perceived differences between these taxons are so minute that markers of differentiation are often unreliable.[28-29] As such, these four species are broadly referred to as the '*donovani* complex.' Analysis of the species has accounted for small genetic differences and has been used to trace disease spread and evolution, however, researchers concluded that not enough genetic variation is present within current available data to warrant this species differentiation. Instead, a corrected differentiation of only two species is proposed:

donovani and *infantum*.[30] While differences in pathology between the two species have been studied, many considerations for rational drug design remain largely similar.[31] Structural characteristics of drug target enzymes are highly conserved across reported species, typically with >95% sequence or structure similarities, as applicable. As such, inhibitors of both species are likely viable through a single drug discovery campaign. However, given similar species with same clinical outcome of VL, cross-validation of known inhibitors on both species is currently limited.

Elements are currently in place to facilitate scalable, target-based, rational drug design toward inhibitors of visceral leishmaniasis. Several proteins of the *Leishmania donovani* complex have been investigated as potential therapeutic targets and, in particular, *N*-myristoyltransferase (NMT) has emerged as a promising one. This target offers multiple design motifs such as *in silico* methods using crystallographic data, enzymatic assay, *in vitro* assays, and availability of *in vivo* disease models. The three screening methods (*in silico*, enzymatic, *in vitro*) may be run in high-throughput or high-content applications, aided by automation to facilitate the screening of large sized compound libraries. Furthermore, techniques relevant to NMT-based drug development may be arranged for *de novo* drug design specifically targeting *L. donovani* NMT, as an additional element of 'target-free'[*] screening to identify an affected target and improve hit optimization, or as a 'piggyback' approach based on existing NMT inhibitors in other species. Despite the availability and scope of these methods, their combined use is surprisingly limited. A discussion of available methods for drug development, their existing applications, and prospective improvements or combinations is presented herein.

[*] 'Target-free,' or phenotypic screening tests potential inhibitors for protozoan growth inhibition and limited human toxicity, irrespective of molecular targets affected.

2.0 *N*-MYRISTOYLTRANSFERASE

Protein myristoylation is an essential function in eukaryotes that occurs via the addition of a myristoyl group to the *N*-glycine terminus of a set of proteins.[32-33] The myristoyl group, derived from myristic acid (*n*-tetradecanoic acid), is a 14-carbon saturated fatty acid serving as a long, hydrophobic tail in a variety of functions, including protein-protein interactions, membrane targeting, signal transduction, and apoptosis.[34-35] The amide bond formation is catalyzed by the enzyme myristoyl-CoA protein *N*-myristoyltransferase (NMT) that occurs as both a post- and, more typically, co-translational modification.[36]

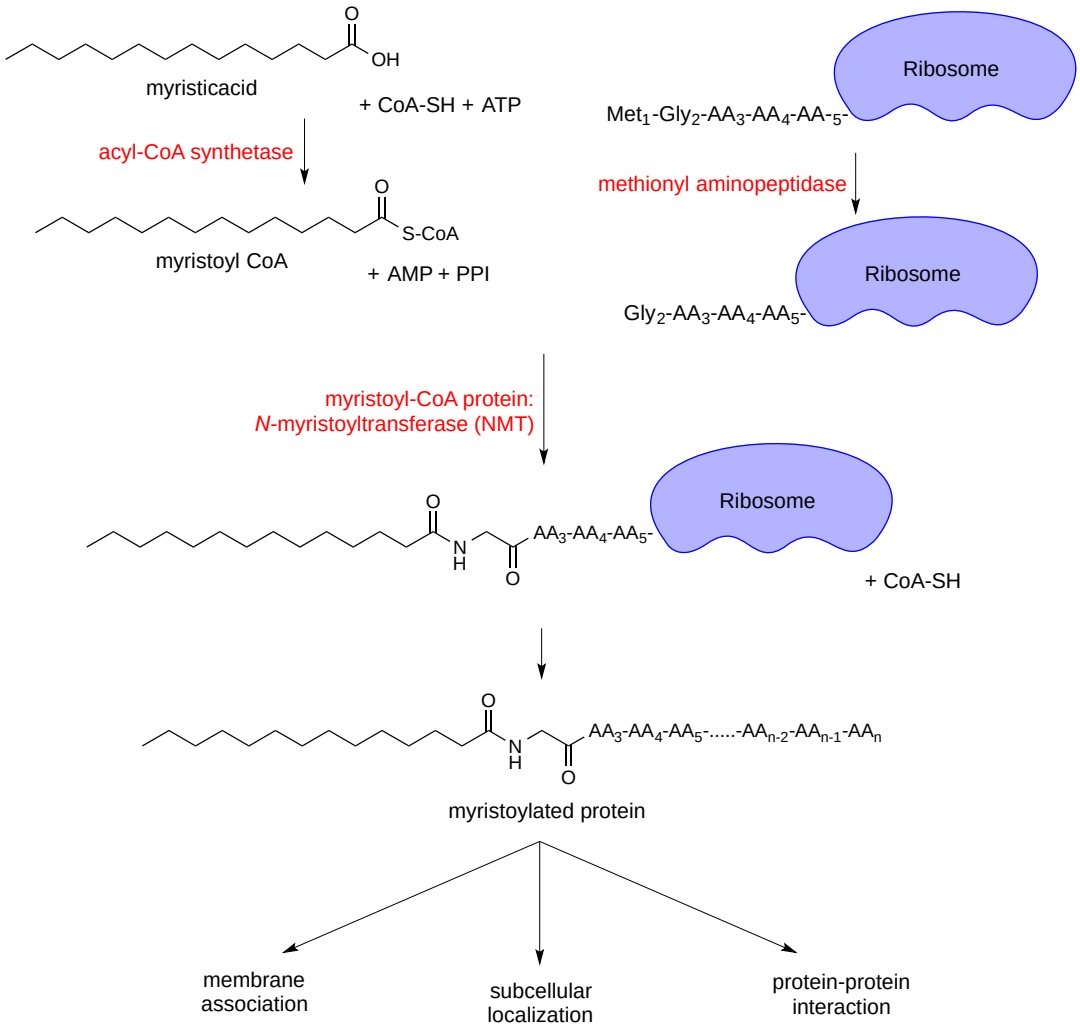


Figure 2: Protein mryistoylation as a co-translational process.
Image used under Creative Commons license.[37]

The binding events required for protein substrate myristoylation have been thoroughly characterized and includes an understanding of substrate specificities for both the myristoyl and protein binding sites. The mechanism proceeds via an "ordered bi bi mechanism," in which myristoyl-CoA first binds to the apo-enzyme.[38] The myristoyl-CoA binding induces an allosteric effect on the enzyme that positions the CoA as a functional part of the protein binding site. The myristoyl group transfer is facilitated by the attack of the incoming N-terminal glycine on the myristoyl-CoA thioester, shown in Figure 2. After the transfer, the free CoA is released first, allowing the enzyme conformation to relax and release the myristoylated substrate protein; the

full catalytic cycle is depicted in Figure 3. As a treatment for disease, cell death is the desired response, achieved through inhibition of the enzyme and disrupting critical cellular processes.

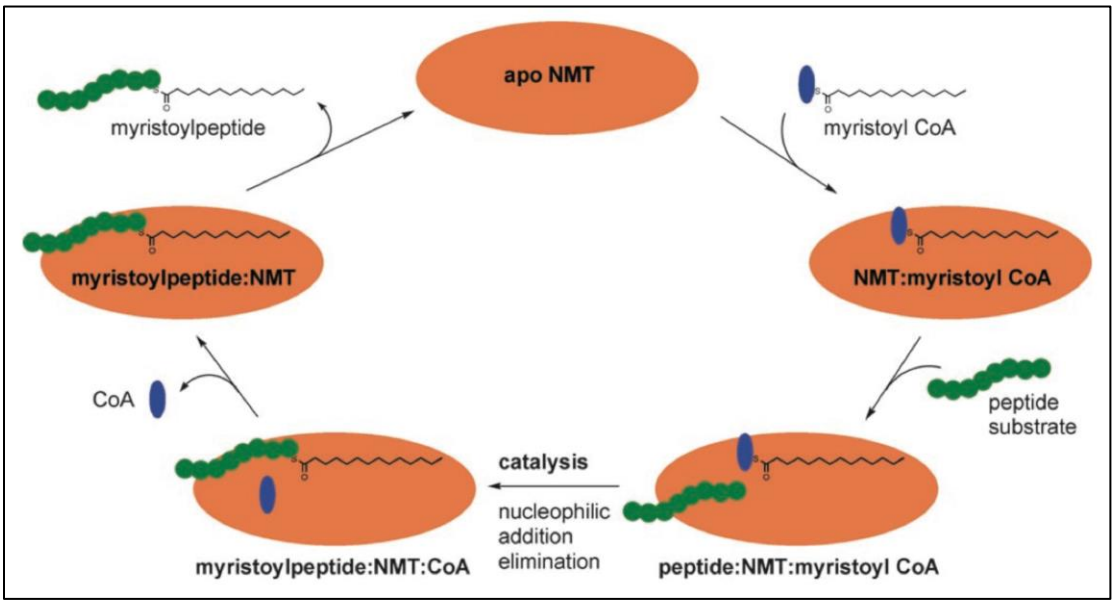


Figure 3: Myristoylation catalytic cycle.
Reprinted with permission from Bowyer, P. W.; Tate, E. W.; Leatherbarrow, R. J.; Holder, A. A.; Smith, D. F.; Brown, K. A., *N*-Myristoyltransferase: A prospective drug target for protozoan parasites. *ChemMedChem* **2008**, *3* (3), 402-408.

2.1 NMT STRUCTURE, FUNCTION, AND SUBSTRATES

The effects of different coenzymes on substrate binding have not been thoroughly studied, but myristate substitutions have been investigated in different species.[39] Generally, specific recognition of myristoyl-CoA has been shown, but the specific acyl-CoA bound to the enzyme has been shown to affect substrate peptide binding. Chain length has been implicated as the major driving force for this specificity, followed by hydrophobicity. For example, 13- and 15-carbon fatty acid substrates have been shown to bind competitively with myristoyl-CoA, but this is largely irrelevant *in vivo*, as these fatty acids are rarely found in eukaryotes.[40] The closest natural/commonly occurring fatty acid, the 16-carbon chain palmitoyl-CoA, can compete with

the myristate binding.[41] While a poor substrate, it is found in much greater intracellular concentrations, yet NMT function is still determined by the myristate. Overall, the enzyme shows little tolerance for alternate acyl-CoA substrates *in vivo*, and the myristoyl-CoA binding site is highly conserved across species studied.

Unlike the myristoyl-CoA binding site, the protein binding site is not highly conserved across species, allowing for substrate specificity for protein myristoylation and species-specific inhibition of the NMT enzyme. Substrate specificity is accomplished by recognition of N-terminal amino acid residues within the substrate protein with as many as 17 terminal residues responsible for substrate recognition.[42-43] These 17 residues are split into three regions; region 1 consists of the first 6 residues, which fit within the binding pocket, and must terminate with a glycine; region 2 consists of the next 6 residues, interacting with the surface of the protein adjacent to the binding pocket, typically consisting of smaller, polar residues; region 3 consists of the final 5 amino acids typically recognized by the enzyme, and is usually comprised of increasingly hydrophilic residues. The enzyme's broader recognition of several substrate residues allows for specificity of proteins to be myristoylated, but the existence of a specific, well-defined binding pocket allows for enzyme inhibition by small molecules. Competitive inhibition of protein myristoylation by small molecule binding to the substrate pocket has been confirmed by kinetic studies, suggesting the viability of NMT inhibition as a therapeutic target.[44-45]

2.2 NMT UTILITY AS A LEISHMANIASIS DRUG TARGET

Several distinctions between mammalian and protozoan NMTs have been identified and

may be exploited for the treatment of protozoan diseases, such as leishmaniasis. Mammals all possess two NMT isozymes, NMT1, and NMT2, each exhibiting slightly different protein substrate specificity, but both are highly similar to one-another and highly conserved across species. For example, human NMT1 (*Hs1NMT*), and *Hs2*NMT share 77% sequence identity. Homologues are, of course, expressed in mice, and the mouse NMT1 and NMT2 share 97% and 96% sequence identity with the respective human isozyme.[46] This sequence homology allows for accurate modeling of NMT inhibition in mouse models of infection.

The genus *Leishmania* only synthesizes one NMT isozyme which is sufficiently different from the human forms to allow for specific inhibition. For example, the *L. donovani* NMT (*Ld*NMT) only shares 42% sequence identity with *Hs1NMT* and highly selective inhibition of *Ld*NMT over both human NMTs has been demonstrated. The NMTs of *leishmania* parasites are highly conserved across different parasite species with *L. major* sharing 97, 96, and 96% sequence identity with *L. donovani*, *L. infantum*, and *L. mexicana*, respectively. Residues associated with the predicted peptide binding groove are fully conserved between *L.* donovani, L. *major*, *L. infantum* and *L. braziliensis.*[47] The combined similarities of parasite NMTs and differences from host NMTs suggest that, despite a focus on treatment of visceral leishmaniasis, expansion of a drug discovery program to successfully combat more forms of the disease may be within reach.

N-Myristoyltransferase inhibition has been demonstrated in a variety of other organisms, typically as a means to treat infectious disease. Closely related to antileishmanial drug development, NMT inhibition is also currently under investigation as a treatment for other trypanosomal diseases.[24, 48-49] Initial determinations of NMT structure and function was performed in yeasts, such as *Candida albicans*, commonly responsible for infection in the

immunocompromised. Several anti-fungal NMT inhibitors have been identified.[50] NMT over expression has also been linked to certain cancers, and NMT enzyme suppression has been proposed as a treatment.[51-53] However, cancer treatments via NMT inhibition is distinct from other NMT inhibitor studies in that cancer cell inhibition would rely on selectivity between the two individual human NMT isozymes, rather than selectivity between human and infection forms. Ultimately, a continually expanding portfolio of known NMT inhibitors offers structure-based insight toward the design of novel inhibitors and starting points for piggyback drug design.

Aside from the enzyme's mechanism and function, a great deal of structural information is known about NMTs beyond basic sequence information. A large number of NMT enzyme structures have been elucidated by X-ray crystallography, most in high resolution (~1.5Å resolution, high degree of completeness). A large majority of the crystal structures include myristoyl-CoA bound to the protein, offering the allosteric conformation relevant for inhibitor binding, rather than the apo-enzyme. Many species include multiple PDB entries, with one structure crystallized with an empty binding pocket, and at least one entry with a known small molecule NMT inhibitor bound, including *Leishmania major* (Figure 4), human NMT1, *Saccharomyces cerevisiae*, and *Candida albicans*.[50, 54-55] With respect to visceral leishmaniasis, the *Leishmania donovani* NMT has been crystallized with a myristoyl-CoA analog (*S*-(2-oxo)pentadecyl-CoA) to avoid hydrolysis, however no structure with a bound inhibitor has yet been crystallized (PDB: 2WUU).[47] Accurate structural data, allows for easily accessible *in silico* studies of protein-inhibitor interaction. Furthermore, known inhibitors bound to the crystal structures serve as means to validate computational docking models. NMT inhibition has been biologically validated as a target in the *Plasmodium* genus.[56] In *L. donovani* NMT is not *fully* biologically validated, as sufficient data regarding myristoylated proteins within the parasite, or

specific downstream effects of NMT inhibition. Nevertheless, NMT has been shown to be an essential enzyme for parasite survival, and inhibition of the enzyme is toxic to kinetoplastid parasites.

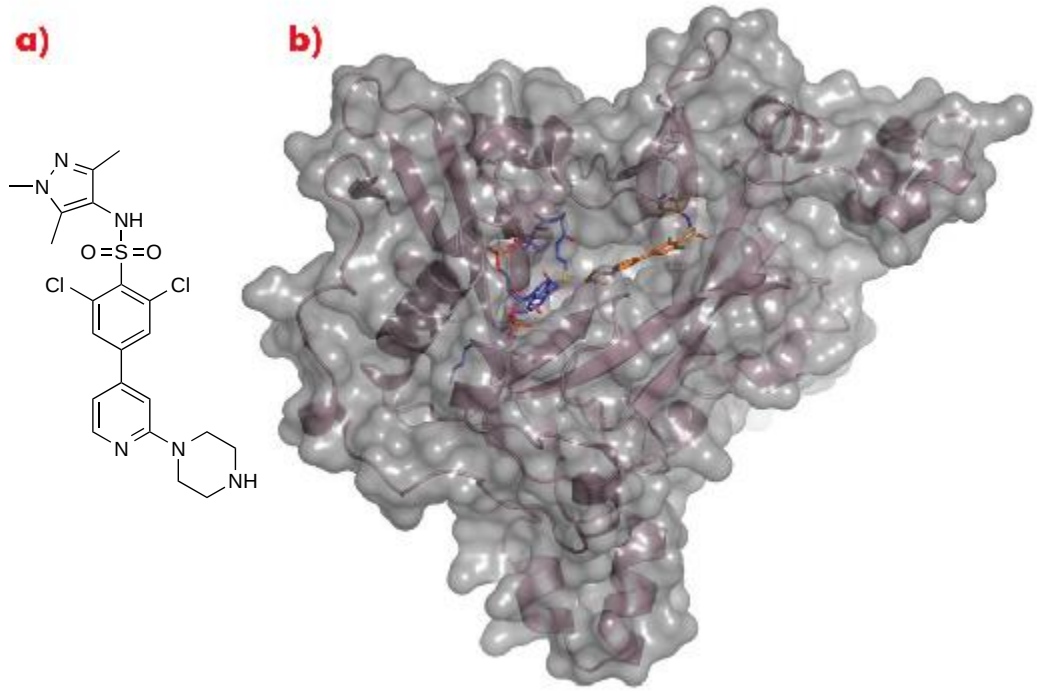


Figure 4: Representative NMT inhibitor and crystal structure.
a) Inhibitor. b) *L. major* NMT crystal structure and computed surface. Myristoyl-CoA (blue C atoms), inhibitor (orange C atoms). Reprinted with permission from David Robinson, University of Dundee.[57]

3.0 SCREENING METHODS

3.1 *IN VITRO* METHODS

A variety of *in vitro* cell culture and analysis methods have been shown as useful model systems for leishmaniasis infection.[58-59] These methods are scalable, enabling large compound libraries to be assayed via high throughput screening.[60] Further analysis of hit compound viability as a lead is possible through related assays, such as toxicity and metabolic stability.

3.1.1 Enzymatic assays

For direct quantification of NMT-specific inhibition, many enzymatic assays are available. Most assays rely on the detection of radio-labeled material incorporated into the product, through techniques such as scintillation proximity assays (SPA).[61] For this assay and others, recombinant NMT proteins can be over-expressed and purified from *E. coli.* Myristoyl-CoA is introduced to the purified protein, wherein the myristoyl group has been modified by tritiation (replacement of hydrogen with radioactive tritium). Instead of a complete protein to be myristoylated, a model peptide substrate is constructed by solid-phase peptide synthesis (SPPS). The sequences of these model peptides are designed based on N-terminal (~8-residue) segments from known protein substrates of NMT. Biotin is applied to the C-terminus of the chain as an affinity tag. Both modified substrates (radio-labeled myristoyl-CoA and biotinylated peptide) are mixed with the

enzyme, and myristoylation occurs. A streptavidin tag held on a solid support encapsulating a scintillant is then added to the reaction. After this addition, the now radio-labeled peptide will migrate to the solid-support as a result of biotin-streptavidin binding, and result in the emission of photons by the scintillant; the full assay scheme is shown in Figure 5. Tritium is well suited for this task, as the path-length of the emitted ß-particle is short enough (1.5 µm) that non-specific emission from unreacted starting material will not reach the scintillation bead, meaning the reaction does not have to be purified before measuring enzyme activity. The emitted photons, and thereby reaction progress, can be monitored via scintillation counter. This process has been used for a wide variety of NMT isozymes, including human NMT1 and 2, *L. donovani*, *L. major*, *T. brucei*, *T. cruzi*, and *P. falciparum*.[24, 48, 62] The process can also be automated through the use of 96- or 384-well plate plates for high-throughput screening.

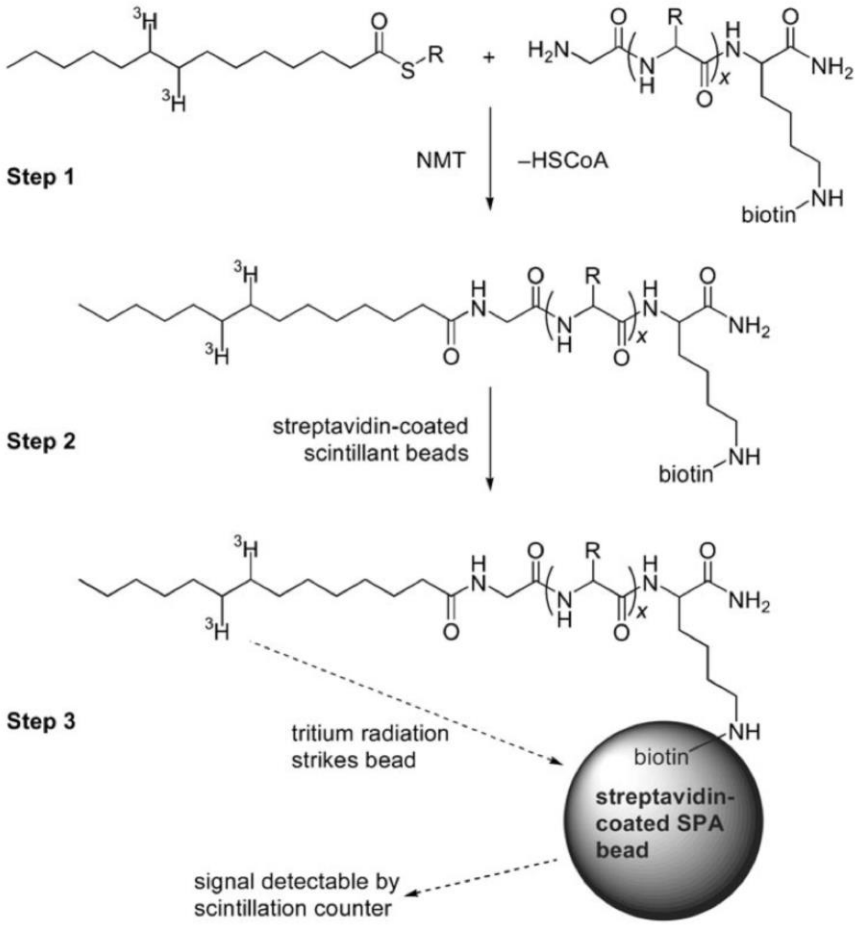


Figure 5: Scintillation proximity assay.
Step 1: Radio-labeleld myristate is added to the biotin-tagged protein mimetic. Step 2: The peptide and radio-label are attached to the scintillant bead by the biotin-streptavidin interaction. Step 3: The radio-label is sufficiently close to the scintillant to cause a photon emission. Reprinted with permission from Bowyer, P. W.; Tate, E. W.; Leatherbarrow, R. J.; Holder, A. A.; Smith, D. F.; Brown, K. A., *N*-Myristoyltransferase: A prospective drug target for protozoan parasites. *ChemMedChem* **2008**, *3* (3), 402-408.

3.1.2 Cellular assay

Several cellular based assays are also available to determine antileishmanial activity. These include culture and inhibition of promastigotes, axenic amastigotes, and intracellular amastigotes. Each of these methods may be used in a target-free screening of inhibitors, but they may also be incorporated with NMT-based drug discovery effort, as NMT is constitutively produced at all stages of parasite development.[48]

Promastigote-based cellular assays are the most straightforward of the three cell-based assays. Promastigotes are grown in a media, potential inhibitors are introduced at suitable concentrations, and growth inhibition is determined.[63] High-throughput applications are available utilizing a plate reader for analysis of cell growth via fluorescent staining, with positive and negative controls (typically amphotericin B, and 1% DMSO, respectively). While promastigotes are often assayed for comparative purposes, their use is waning, as techniques for the high-throughput screening of more biologically relevant parasite forms has become commonplace.

Axenic amastigote screening provides a more biologically relevant assay than promastigotes, since the former is the active parasite found within host mammals.[64-65] Screening is accomplished much the same way as with promastigotes in terms of controls, and hit identification by fluorescence. However, the actual growth of the parasites differs, as they must be chemically induced to continue multiplying as amastigotes, rather than revert to the promastigote form. This is accomplished via manipulation of pH to mimic macrophage conditions, and occasionally by the inclusion of 5% CO_2 in the atmosphere, or incubation of cells at biologically relevant temperatures (37 ºC).

Screening compounds against intracellular amastigotes provides the best *in vitro* similarity to conditions found *in vivo*.[66-67] In this method, promastigote cultures are allowed to infect macrophage differentiated host cells in growth media. After a period of initial infection, the non-internalized promastigotes are rinsed away with a buffer, and the parasites are allowed to continue to grow under the presence of test compounds, or controls. Chemical differentiation of the host cells into macrophages is required for two reasons - first, the macrophage best mimics the *in vivo* context for a host infection, and second, without differentiation to non-dividing macrophages, parasitized cells would be overrun by replicating cells. THP-1 (human acute

leukemia monocyte) cells are most often used for this purpose. Determination of inhibitor activity is accomplished by visualization with a fluorescent DNA-binding stain. Areas rich in DNA will fluoresce, predominantly the THP-1 cell nucleus, and parasite kinetoplasts. The difference in size between these two structures may be exploited for high-content screening. One assay yields not only a ratio of parasites to host cells, but also a quantification of host cells in each sample well to serve as a measure of toxicity induced by the compounds. These measurements can be taken by automated fluorescence microscopy, but require a software suite for the automated/accurate image analysis.[68]

3.1.3 Secondary screening

After hit compounds are identified, several other *in vitro* methods have been used to assist in lead identification as secondary screens. If not part of a the primary screen, assay hits may be tested independently against uninfected THP-1 cells to determine toxicity. Metabolic stability is also an important aspect for lead compound identification and optimization, as even the most potent hit is ineffective if metabolized too quickly.[69] Preliminary metabolic data may be gathered by subjecting the test compound to either rat or human liver microsomal stability assay. As a final consideration in lead identification and optimization, hit compounds may be screened against cytochrome P450, to avoid potential drug-drug interactions.[70]

3.1.4 Screen analysis and validity

Large scale screening methods must provide a reliable means to identify biologically active compounds from a large compound library, with assays performed singly (rarely in duplicate or

triplicate). A statistical method for determination of assay quality has been proposed, yielding a dimensionless coefficient called the Z-factor, or Z-score (Equation 1).[71] A Z-factor of 1 indicates an ideal assay, with standard deviations of zero, and infinite dynamic range. Typically Z-factors between 1 and 0.5 indicate a good quality assay, with a large separation band between hits and inactive compounds.

$$Z\ factor = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

Equation 1: Z-Factor for HTS validity
σ: standard deviation, μ: mean, p: positive control, n: negative control

3.2 *IN VIVO* MODELS OF VISCERAL LEISHMANIASIS

After identification and optimization of hit compounds through *in vitro* methods, animal models are required to better mimic infection and treatment in mammalian hosts. For developing new chemotherapies, i*n vivo* models serve to confirm compound activity determined from cellular and enzymatic assays, as well as provide information regarding administration routes, distribution, metabolism, excretion, and toxicity (ADMET). Several species have been studied as hosts for the *L. donovani* parasite as models for visceral leishmanias.[59] Rodents, canines, and non-human primates have all been used for this task.

Rodent models are the most accessible models of *L. donovani* infection of a mammalian host. A variety of species have been studied, including C57BL/6 mouse, and Syrian golden hamster, but the BALB/c mouse model is most common.[72] Canine models of the disease have also been used in studies of parasite biology, but with little emphasis for use on development of chemotheraputics.[59] Establishing canines as accurate models is important nonetheless, as they

have been shown to exhibit closer disease pathology to humans than available in rodent models. Also, the study of leishmaniasis in canines is useful to determine their role as host reservoirs for certain species of the parasite.[73] Finally, non-human primates have been studied as the most advanced, human-like model of leishmaniasis. Many species of primate are quite resistant to *Leishmania* infection, however, the languor (*Presbytis entellus*) has been identified as a promising model system for human leishmaniasis infection. The languor is susceptible to a lasting VL infection, ultimately resulting in death, and displays the same pathogenesis and immune response as in humans, allowing for their use in the study of antileishmanial vaccine candidates.[74]

4.0 LIMITED STATE OF ANTILEISHMANIAL DEVELOPMENT

A wide array of experimental tools are available for the study and development of leishmania-specific NMT inhibitors, yet there is a surprising lack of synergy and cross validation among these models. Currently, target-free screening is gerally preferred to target-based design: many putative targets besides NMT are characterized (albeit in less detail), and target-free screening allows for testing compounds 'holistically' focusing on overall efficacy, independant of knowledge about a particular molecular target.[65-66, 69, 75-77] Choice of parasitic form (promastigote, and axenic or intracellular amastigote) is non-trivial, but improvements in high-throughput assays for the later stages of the life cycle have rendered promastigote assays fairly obsolete.[67, 78] Axenic and intracellular amastigote models are more biologically relevant, and are available in for use in high-throughput applications. To date, several target-free HTS/HCS based screens for *Leishmania* inhibitors has been performed, with z-scores > .5, indicating good quality screens.[79] These efforts have resulted in the identification of novel scaffolds, but no attempt has been made to associate hits with a specific target (either by *in silico* methods, or enzymatic assay).

One NMT-based HTS has been performed utilizing the scintillation proximity assay, against the Pfizer global representative library (~150,000 compounds).[62] This screen successfully demonstrated the ability to selectively inhibit *Ld*NMT over *Hs1*NMT (Figure 7), and identified several scaffolds for NMT inhibition, representatives shown in Figure 6. However, this screen

was not without shortcomings, such as a lack of testing in cellular assays to confirm bioactivity and selectivity of identified hits. Also, while each scaffold generated a series of 'local' hits, only inhibition data for top hits within each family was reported, rather than observed SAR.

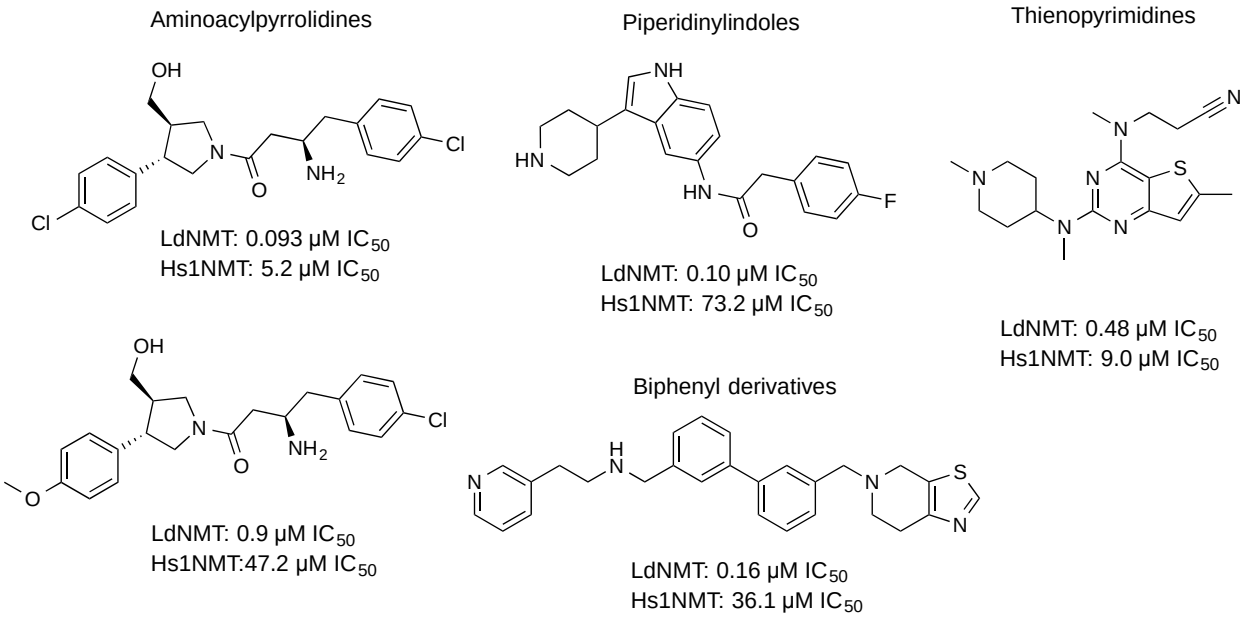


Figure 6: *Ld*NMT assay hits and selectivities.

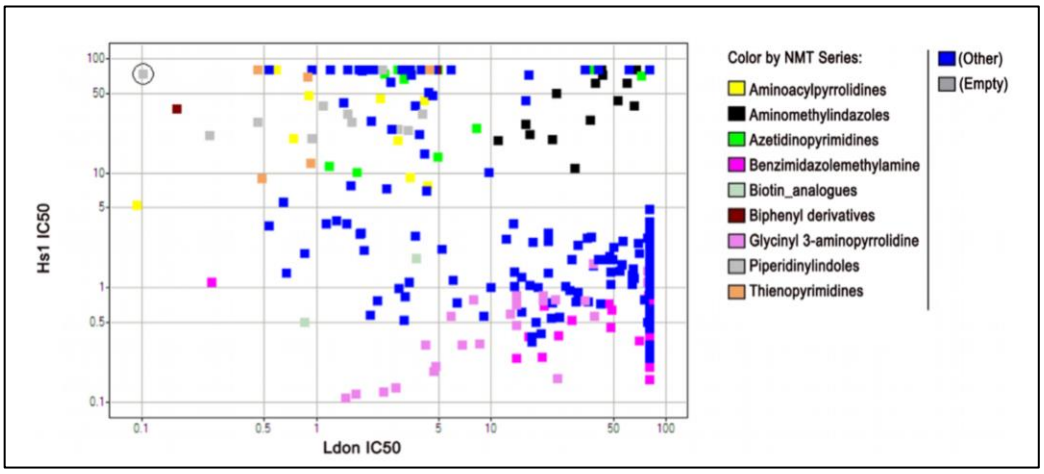


Figure 7: Tunable selectivity between *Ld* and *Hs1* NMT inhibition.
Reprinted under Creative Commons license from Bell, A. S.; Mills, J. E.; Williams, G. P.; Brannigan, J. A.; Wilkinson, A. J.; Parkinson, T.; Leatherbarrow, R. J.; Tate, E. W.; Holder, A. A.; Smith, D. F., Selective inhibitors of protozoan protein *N*-myristoyltransferases as starting points for tropical disease medicinal chemistry programs. *PLoS Negl Trop Dis* **2012**, *6* (4), e1625.[80]

In silico analysis of antileishmanial NMT inhibitors is also limited. Despite the large

quantity of crystallographic data available for *Leishmania* spp. NMT enzymes, few detailed *in silico* docking or modeling studies have been published. Of the few studies available, many are not specific to NMT, but rather, focus on a cross-sectional docking study of inhibitors against a range of potential targets. Ogungbe, *et al.* have published two separate reports docking plant-based phenolic compounds (stilbenoids, phenylpropanoids, flavonoids, quinones, etc) to a large series of crystal structures to determine potential targets.[81-82] In either instance, a library of chemicals (some of which have shown broad antiparasitic activity) is docked to the array of structures, and energies reported. However, neither report offers a more in-depth analysis of the data, by way of rationalizing existing cellular assay data with docking results, or collaborative work to screen top computational hits against cellular or enzymatic assays.

Sobarzo-Sanchez *et al.* have studied a small series of oxoisoaporphine antileishmanials (Figure 8) in detail, providing *in vitro*, *in vivo*, and *in silico* analysis.[83] Their aim was similar to Ogungbe's work, but more successful in identifying potential targets. Utilizing 128 known *Leishmania* enzymes with docked ligands as the basis for a training dataset, the group utilized bioinformatic analysis (via 'MARCH-INSIDE' software) to determine 4 putative targets, including NMT. From these results, an NMT enzymatic assay could easily confirm oxoisoaporphines as NMT inhibitors, and provide a positive or negative training dataset for further *in silico* study of oxoisoaporphines.

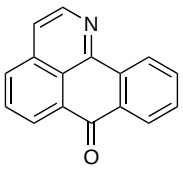


Figure 8: Oxoisoaporphine scaffold.

While NMT has been studied in leishmaniasis, and its inhibition has been shown as a means to combat parasite growth, the discovery of corresponding *Leishmania* NMT inhibitors is

limited. Simple efforts to utilize existing methods synergistically can increase the efficiency in which NMT inhibitors are discovered and optimized.

5.0 NMT STUDIES IN RELATED ORGANISMS

As described previously, NMT enzyme activity has been proposed as a target for a variety of fungal and protozoan diseases, and a number of compounds have been reported to inhibit the NMT enzyme and disease growth. However, in comparison to *Leishmania* NMT inhibitors, NMT inhibition in other species has been studied in much greater detail, with inclusion of SAR, *in silico,* and crystallographic supporting data. Some relevant data with regard to cross-inhibition of *Leishmania* NMTs is occasionally discussed within these studies, but is rarely presented in context of SAR studies focusing on improving antileishmanial potency. It is not uncommon for large swaths of medicinal chemistry data to go unreported in scientific journals and only published in patents.[84] This is no different for NMT inhibitors: while there is a lack of SAR data and discussion for antileishmanials presented within journal articles, certain data-sets can be found in related patents, but without relevant discussion. A variety of compound classes have been reported as NMT inhibitors in other species with some crossover studies on *Leishmania* spp, including benzofurans and similar heterocycles, pyrazole sulfonamides, quinolines, and peptides/peptidomimetics. These scaffolds and associated analyses can serve as starting points for piggyback drug development strategies.[85]

5.1.1 Benzofurans

Benzofurans, and associated compounds (benzothiazoles, benzothiophenes, indoles) are amongst the most studied NMT inhibitors, and this broad group of compounds has been used for piggyback hit generation/optimization across several species.[86-94] QSAR studies have been performed on existing hit datasets to determine pharmacophore models, which have been computationally used to generate new prospective hits.[95-96] In terms of activity-based SAR, a piggyback approach using the same or similar heterocyclic core is common amongst other types of disease, which is visually apparent between studies on different species.[97-98] Despite the core similarities between inhibitors, each subsequent study has expanded around existing SAR (species specific), in continually good detail.[99] Furthermore, many papers include crystallography data showing the binding pose of their top hit(s). However, no journal articles discuss the implications of these results with respect to the development of antileishmanials.

Benzofurans and furan-like compounds have been patented for use as NMT inhibitors of *P. falciparum,* and *L. donovani.* Despite a lack of reporting in primary literature sources, the patent features a SAR of 140 compounds with similar heterocyclic cores, and relevant IC_{50} concentrations (SPA or fluorescence assay) for both *L. donovani* and *P. falciparum.*[100] Of the compounds screened, 74 have been identified by the authors as 'hits' (< 5 μM IC_{50}) against *Ld*NMT. The most potent, shown below (Figure 9), possesses an IC_{50} of 0.01 μM. Unfortunately this hit was not screened against human NMT1 to determine selectivity. The most potent inhibitor with selectivity data displays 0.027 μM IC_{50} against *Ld*NMT, and 1.00 μM IC_{50} inhibition against *Hs1*NMT, roughly 40-fold selectivity favoring parasite isozyme inhibitions.

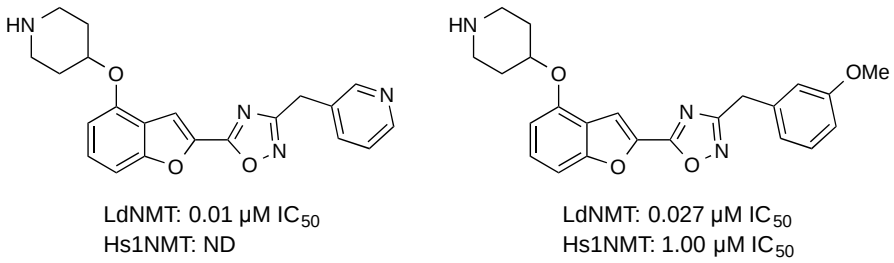


Figure 9: Benzofurans as NMT inhibitors.

The most recently published study of this class of compounds investigated the use of substituted benzothiophenes as inhibitors of *P. falciparum* and *P. vivax* (Figure 10). The study proposes 'ligand efficiency dependent lipophilicity' (LELP) as a single metric to assist in triage of molecules, and as a strong predictor of drug-likeness, utilizing compounds from the previous patent. Included are crystal structures of two inhibitors bound to *P. vivax* NMT, displaying complementary binding modes to previous characterizations. LELP optimization resulted in ~100-fold increase in enzyme affinity.[101]

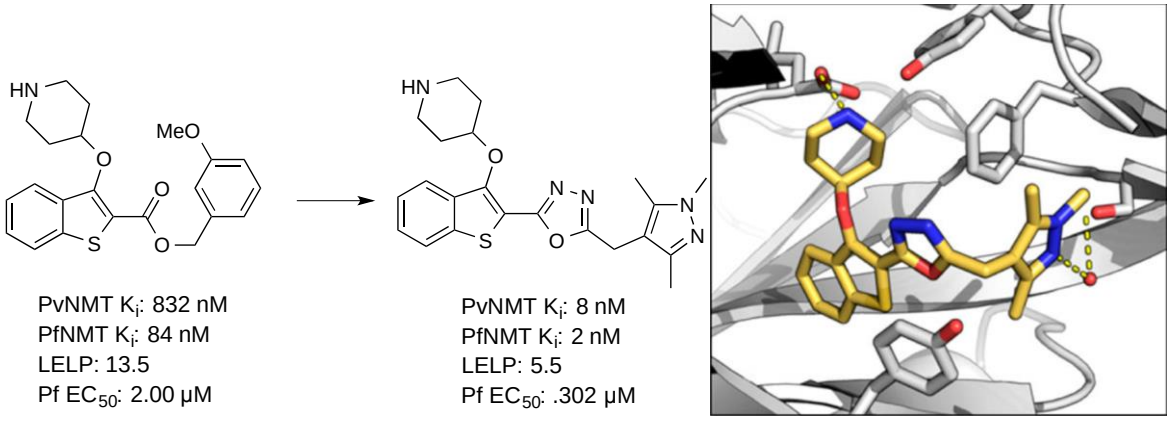


Figure 10: LELP-optimized *Plasmodium* inhibitors and crystal structure with ligand.
(PDB: 4CAF) Reprinted with permission from Rackham, M. D.; Brannigan, J. A.; Rangachari, K.; Meister, S.; Wilkinson, A. J.; Holder, A. A.; Leatherbarrow, R. J.; Tate, E. W., Design and synthesis of high affinity inhibitors of *Plasmodium falciparum* and *Plasmodium vivax N*-myristoyltransferases directed by ligand efficiency dependent lipophilicity (LELP). *J Med Chem* **2014**, *57* (6), 2773-2788. Copyright 2014 American Chemical Society.

LELP is calculated as cLogP/LE. Ligand efficiency, LE = $[-RT\log(K_i)]/(\text{no. of heavy atoms})$. K_i values are calculated from the experimentally determined IC_{50} values, the substrate

concentration ([S]), and the Michaelis–Menten constant (K_m) as described by the Cheng–Prusoff equation (Equation 2).[102] K_m values are used from previously experimentally determined constants.

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$$

Equation 2: Cheng-Prusoff equation
[S] = substrate concentration

5.1.2 Pyrazole sulfonamides

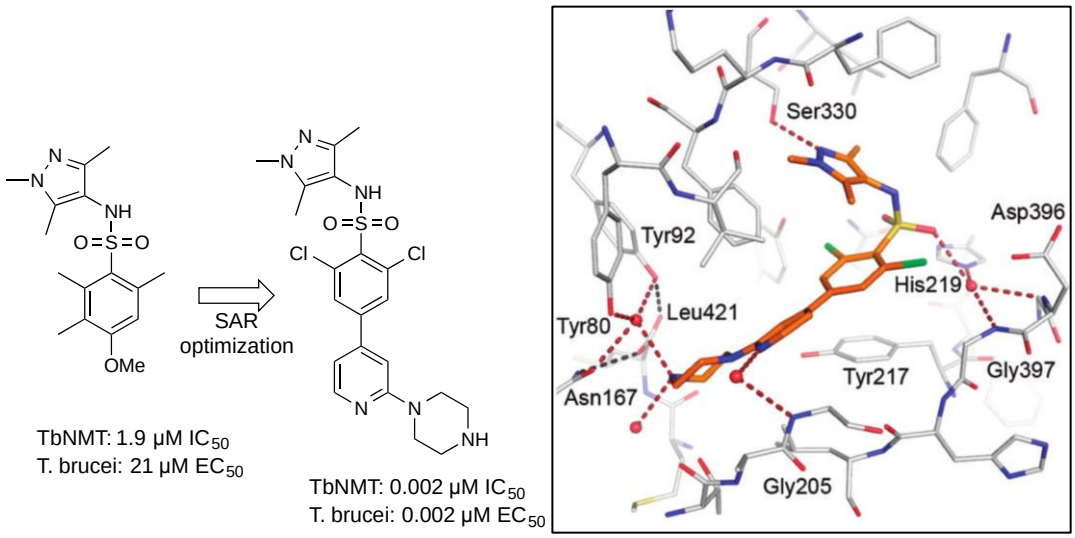


Figure 11: Pyrazole sulfonamide NMT inhibitors and representative crystal structure.
Reprinted with permission from Brand, S.; Cleghorn, L. A.; McElroy, S. P.; Robinson, D. A.; Smith, V. C.; Hallyburton, I.; Harrison, J. R.; Norcross, N. R.; Spinks, D.; Bayliss, T.; Norval, S.; Stojanovski, L.; Torrie, L. S.; Frearson, J. A.; Brenk, R.; Fairlamb, A. H.; Ferguson, M. A.; Read, K. D.; Wyatt, P. G.; Gilbert, I. H., Discovery of a novel class of orally active trypanocidal *N*-myristoyltransferase inhibitors. *J Med Chem* **2012**, *55* (1), 140-152. Copyright 2011 American Chemical Society.

Pyrazole sulfonamides are less ubiquitous than benzofuran-like molecules for NMT inhibition, but nevertheless, extensive SAR studies have been performed towards the inhibition of *T. brucei*, and *T. cruzi* parasites.[103-104] Screening of ~60,000 compounds yielded an initial hit, and subsequently over 200 analogs were synthesized for further analysis.[103] The initial hit, its

binding pose in a crystal structure, and optimized compound are shown in Figure 11. Studies on these analogs include corresponding selectivity data between *Tb*NMT and *Hs1*NMT via SPA assay, and *in vitro* activity against *T. brucei* parasites. Beginning with an initial hit of 21 μM (IC_{50}) against the *T. brucei* parasite, the optimizations culminated in a compound with 0.002 μM potency. The paper indicates cross-inhibition of *L. major*, however only inhibition by the optimized compound (optimized specifically for *T. brucei* inhibition) has been reported (*Lm*NMT IC_{50}, 0.002 μM), rather than data from the series of compounds.

Included in the study, 5 crystal structures with bound pyrazole sulfonamides inhibitors are reported. However, *T. brucei* crystallization has not been reported, and the authors utilized *Lm*NMT as a surrogate model for *Tb*NMT, but did not discuss SAR implications of these compounds regarding antileishmanial development. Nevertheless, a wide range of analogs has been studied, and a close correlation between NMT inhibition and *T. brucei* parasite inhibition has been established (Figure 12).

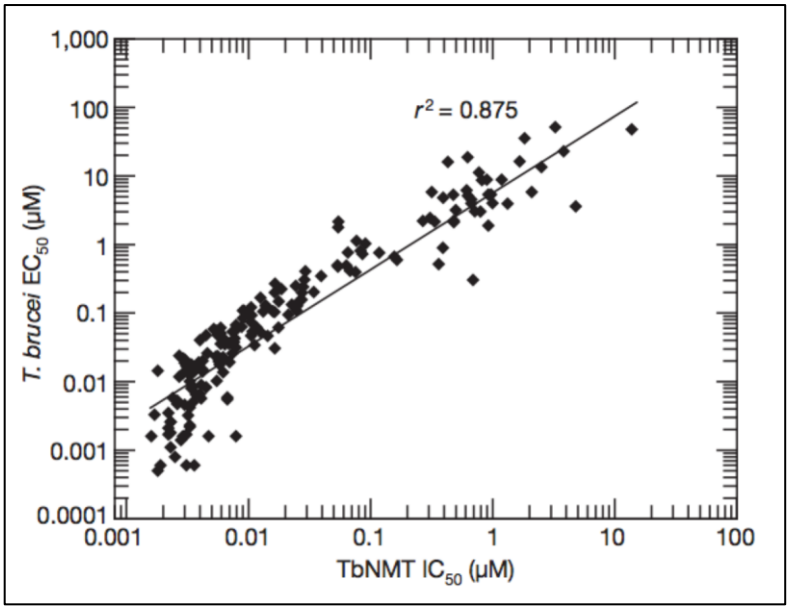


Figure 12: *Tb*NMT inhibition correlates to *T. brucei* parasite inhibition.

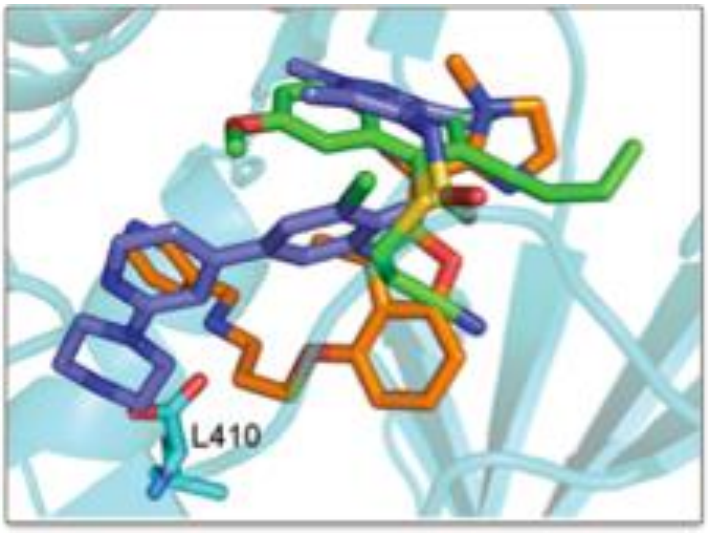
Image used with permission from Frearson, J. A.; Brand, S.; McElroy, S. P.; Cleghorn, L. A.; Smid, O.; Stojanovski, L.; Price, H. P.; Guther, M. L.; Torrie, L. S.; Robinson, D. A.; Hallyburton, I.; Mpamhanga, C. P.; Brannigan, J. A.; Wilkinson, A. J.; Hodgkinson, M.; Hui, R.; Qiu, W.; Raimi, O. G.; van Aalten, D. M.; Brenk, R.;

Gilbert, I. H.; Read, K. D.; Fairlamb, A. H.; Ferguson, M. A.; Smith, D. F.; Wyatt, P. G., *N*-Myristoyltransferase inhibitors as new leads to treat sleeping sickness. *Nature* **2010**, *464* (7289), 728-732.

Much like the benzofuran NMT inhibitors, the series of pyrazole sulfonamides inhibitors has also been patented.[105] Similar to benzofurans, the pyrazole sulfonamides have been tested against *Lm*NMT via SPA assay, as described in the patent, yet these results are unreported in the original paper. Inclusion of *Lm*NMT inhibition data is far more limited than corresponding *Tb*NMT data - only ~40 compounds of the total screen were also assayed for *Lm*NMT inhibition.

5.1.3 Quinolines

Recently, a novel series of quinoline compounds has been discovered to inhibit *P. vivax* via high throughput screening. Structurally similar hits within the original screening set were tabulated, and yielded a concise SAR. This study culminated in a *Pv*NMT inhibitor with a 2.9 μM IC_{50} in the enzymatic assay. A binding mode has also been elucidated via crystallography (PDB: 4A95, Figure 13). In comparison with other known inhibitors of *Ca*NMT and *Tb*NMT, the quinoline series occupies a slightly different area of the binding pocket.



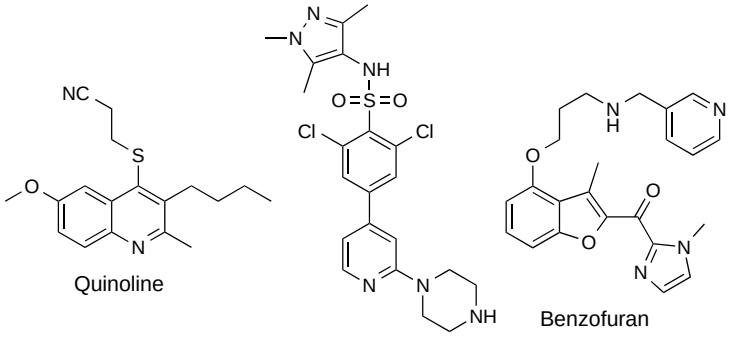


Figure 13: Structure overlay of inhibitor species.
Quinoline shown in green, benzofuran in orange, pyrazole sulfonamide in blue. Image used with permission from Goncalves, V.; Brannigan, J. A.; Whalley, D.; Ansell, K. H.; Saxty, B.; Holder, A. A.; Wilkinson, A. J.; Tate, E. W.; Leatherbarrow, R. J., Discovery of *Plasmodium vivax N*-myristoyltransferase inhibitors: Screening, synthesis, and structural characterization of their binding mode. *J Med Chem* **2012**, *55* (7), 3578-3582. Copyright 2012 American Chemical Society.

5.1.4 Peptide/peptidomimetics

Peptide-based and peptidomimetic NMT inhibitors have been reported for *C. albicans*, and *P. falciparum.*[106-109] Detailed SAR is more limited than for small molecule inhibitors, yet activity is similarly impressive, with as low as 0.04 μM IC_{50} against *Ca*NMT. Notably, peptide and peptidomimetic inhibitors can take advantage of the high degree of NMT substrate specificity, with a maximum selectivity of 2200-fold demonstrated in *Ca*NMT over *Hs1*NMT. Structural

characterization of a peptide bound to *S. cerevisiae* (PDB: 1IID) has been achieved, and analysis of >100 synthetic peptides has been performed to determine preferred amino acid composition for binding affinity along an 8 residue synthetic peptide.

6.0 FUTURE PROSPECTS IN ANTILEISHMANIAL DEVELOPMENT

Existing infrastructure for design, synthesis, and screening of *L. donovani* inhibitors can be better utilized for NMT-inhibitor-based drug discovery. A simple, comprehensive improvement to existing antileishmanial design would be the addition of NMT assay screening of target-free hits. By testing a greatly reduced number of compounds, a minimal amount of complexity is added to an existing screening campaign. Compounds that do not inhibit NMT may be studied and optimized as with any other target-free screening. Targets shown to inhibit NMT activity may be better optimized using target-based methodologies. In either case, further use of secondary screening can be carried out to better identify lead-like compounds.

The relevance of a target-based drug development methodologies for antileishmanials warrants discussion.[110-112] Historically, the increase in computational power, and knowledge of genetics and proteomics has made target-based screening *appear* as an encouraging method for drug discovery. However, in practice, target-based drug design involves assumptions about mechanism of action, identification and validation of a drug target, and sometimes complex interactions between a disease and the body. Target-free screening avoids these pitfalls by exclusively focusing on the observed drug efficacy. In the case of *N*-myristoyltransferase as a target for visceral leishmaniasis treatment, the enzyme sidesteps these barriers. Crystal structures with known inhibitors and enzymatic assay provide an immediate link to compare results of the two methods between themselves, and between data associated with cellular assays. Transgenic

parasites null for the NMT-encoding gene in both *L. major* and *L. donovani* have demonstrated the necessity for NMT enzyme expression. The parasite biology with respect to NMT inhibition is well understood, and selective inhibition over human isozymes has been demonstrated. As an additional metric for compound screening and triage, LELP considerations may be applied to compound-sets. Overall, NMT presents itself as a well characterized enzyme, suitable for target-based development.

Optimization of existing NMT inhibitors via a piggyback approach is the most immediately accessible option for antileishmanial development. Existing SAR and crystallographic data for other species provide a comparable framework for antileishmanial design and synthesis.

Peptide and peptidomimetics, in particular, offer a unique route to *Leishmania* inhibition, with the potential for high specificity toward *Ld*NMT over *Hs*NMT, minimal toxicity, and easy library synthesis of analogs via peptide coupling methods. Kinetic and inhibition studies of known NMT-inhibiting synthetic peptides and peptidomimetics on *L. donovani* NMT may be a first step, considering similarities between studied yeast NMT and *Leishmania* NMT. Significant differences between the proteins occur on the opposite face of the protein and CoA binding sites, and these differences are not purported to alter protein function, offering direct comparison of bound *Sc*NMT inhibiting protein with the *Lm*NMT crystal structure (Figure 14).[47] Secondary screening of drug metabolism is also necessary to help overcome rapid metabolism commonly associated with peptide based drugs.[113]

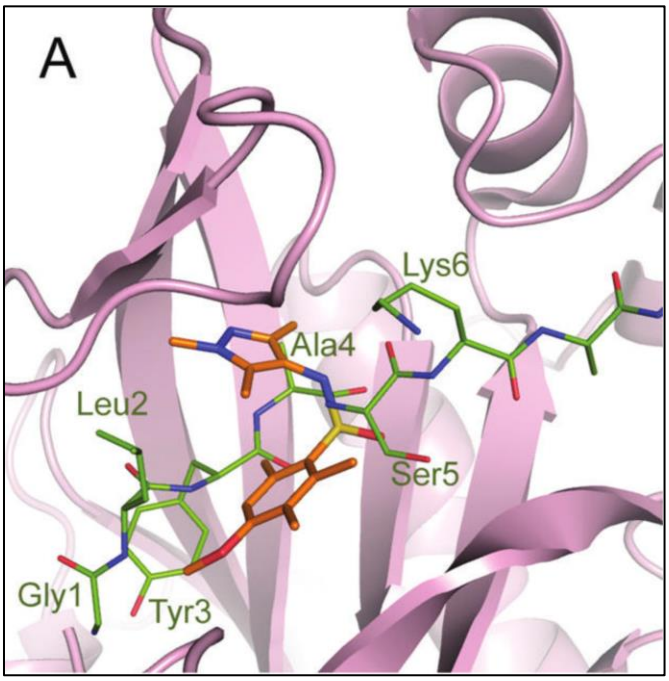
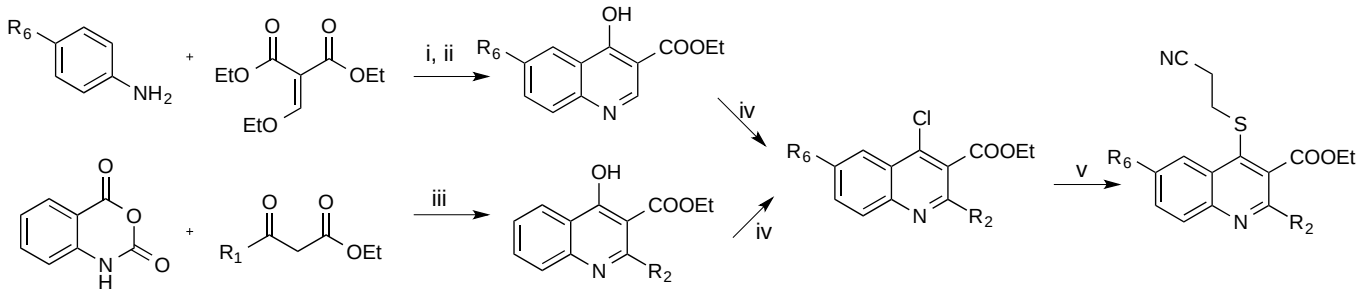


Figure 14: Overlay of peptide and small molecule NMT inhibitors.
Peptide (green) from *Sc*NMT crystal structure (PDB: 1IID), inhibitor (orange) and enzyme (pink) from *Lm*NMT crystal structure (PDB: 4A2Z). Image used with permission form Brand, S.; Cleghorn, L. A.; McElroy, S. P.; Robinson, D. A.; Smith, V. C.; Hallyburton, I.; Harrison, J. R.; Norcross, N. R.; Spinks, D.; Bayliss, T.; Norval, S.; Stojanovski, L.; Torrie, L. S.; Frearson, J. A.; Brenk, R.; Fairlamb, A. H.; Ferguson, M. A.; Read, K. D.; Wyatt, P. G.; Gilbert, I. H., Discovery of a novel class of orally active trypanocidal *N*-myristoyltransferase inhibitors. *J Med Chem* **2012**, *55* (1), 140-152. Copyright 2011 American Chemical Society.

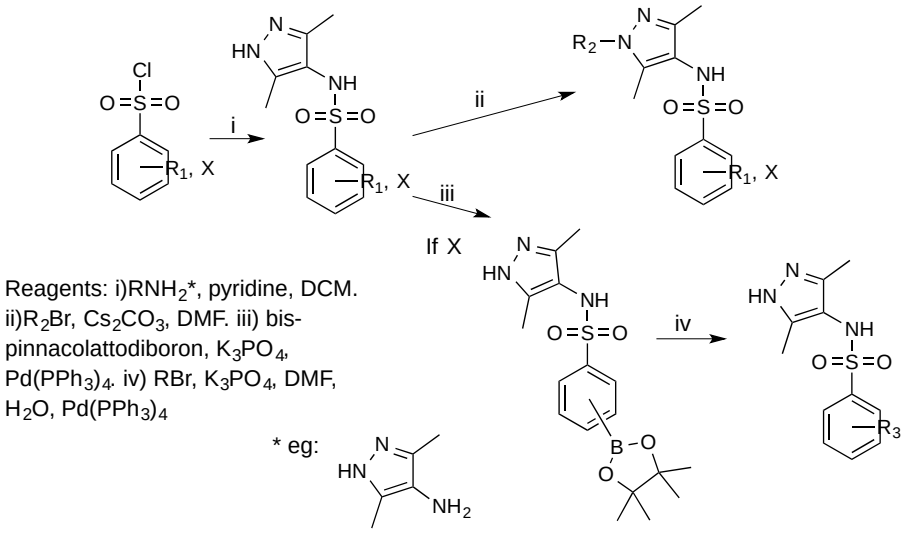
Quinoline-based NMT inhibitors may also serve as a promising source of piggyback development candidates. Initially, screening of the *P. vivax* hits against *L. donovani* NMT can provide the first step to understanding the efficacy of this class of compounds against the *Leishmaniases.* Synthesis of these quinolines is well established to allow for further SAR studies and optimization.[88] Notably, the nitrile-bearing thioether is attached last, allowing for relatively easy synthesis of relevant analogs for SAR studies (Figure 15). Visually, the binding pocket is not fully utilized by the quinoline analogs, but the nitrile group shares some overlap with other inhibitor classes. Replacement of the nitrile group with other potent pharmacophores may yield an increase in both activity and selectivity by utilizing a larger surface of the binding site.



Reagents and conditions: i) 140 ºC, 1hr. ii)Ph_2O, reflux, 2 hr. iii) NaH, *N,N*-dimethylacetamide, 120 °C, 20 min. iv) $POCl_3$, 110 °C, 20 min. v) 3-mercaptopropanenitrile, K_2CO_3, THF, reflux 4hr.

Figure 15: Quinoline synthetic scheme.

Existing SAR and synthetic routes to benzofurans, benzothiophenes, and pyrazole sulfonamides are extremely well established, and offer insight towards synthesis of new analogs. Synthetic routes to these compounds are relatively simple, with numerous points to facilitate combinatorial synthesis, or substitution of bioisosteric fragments. For example, pyrazole sulfonamides are accessible in minimal steps, with orthogonal steps to introduce additional moieties or substitutions (Figure 16).[103]



Reagents: i)RNH_2*, pyridine, DCM. ii)R_2Br, Cs_2CO_3, DMF. iii) bis-pinnacolattodiboron, K_3PO_4, $Pd(PPh_3)_4$. iv) RBr, K_3PO_4, DMF, H_2O, $Pd(PPh_3)_4$

Figure 16: Pyrazole sulfonamide synthesis.

Inhibitors specific to *L. donovani* NMT have been identified via high throughput enzymatic assay. Re-assay of structurally similar hits can provide a direction for future SAR

study, and synthesis of analog compounds is the next logical step for development and optimization of these compound classes. Synthetic routes to these specific compounds are not available via literature methods, but adaptations of existing methodologies should suffice. Biphenyl derivatives are readily synthesized from Suzuki-coupling of substituted aryl groups.[114] Piperidinylindoles are easily synthesized from a corresponding indole starting material.[115] Chiral acylpyrrolidones are commercially available with a variety of aromatic substitutions and other pyrrolidines may be synthesized using a variety of established procedures.[116-119] Notably, 3,4-disubstituted pyrrolidines may be diastereoselectively synthesized from corresponding ethyl cinnamate analogs,[120] followed by mild, selective N-demethylation.[121] Subsequent coupling with commercially available, or readily synthesized, amino-substitued fragments by amide bond formation complete the full analog synthesis (Figure 17). Similarly substituted thienopyrimidines have been synthesized as treatments for hematological malignancies and as calcium channel blockers (Figure 18).[122-124] In all cases, synthetic methods allow for easy introduction of structural variety, allowing for combinatorial synthesis of a variety of analogs.

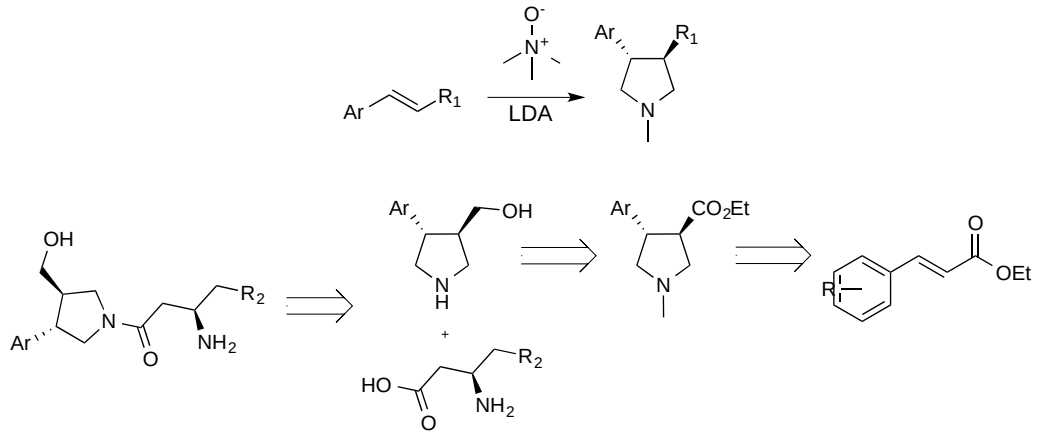


Figure 17: Pyrrolidine synthesis and acylpyrrolidine retrosynthesis.

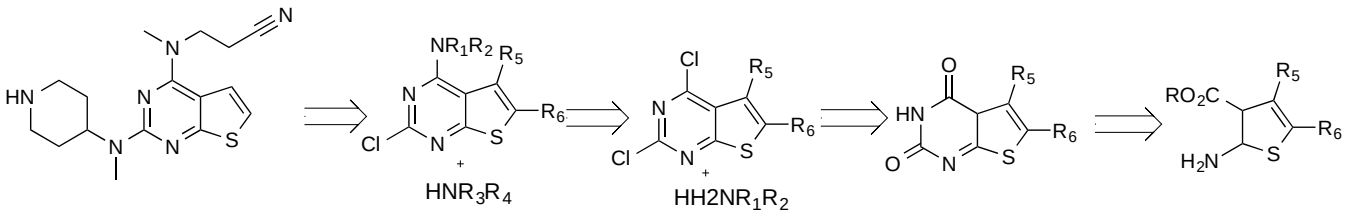


Figure 18: Thienopyrimidine retrosynthesis.

Finally, purely *in silico* methods may provide the easiest route for screening large compound libraries. While there is currently a lack of computer based drug design toward antileishmanials, selecting NMT as a target offers a simple, robust approach for development. Preliminary screening in silico can be accomplished through tandem screening of compounds against *Leishmania* and human NMT isozymes. This can be done with relatively little computing power (dedicated desktop as opposed to multicore servers) and at low cost (several free docking programs offer comparable docking performance to commercial software suites).[125-126] Any molecular library may be screened, with the substantial ZINC database being an excellent starting point.[127] Docking may be validated based on comparison with ligand positions in crystal structures, and by NMT enzyme assay of top hits. Likewise, existing SAR studies may be applied to provide extensive training datasets for validation of *in silico* methods. Computational methods may be inserted at any point of NMT-based antileishmanial discovery to achieve an optimal relationship between screening, validation, and generation of new compounds to test.

7.0 CONCLUSIONS

The global disease burden and lack of sufficient clinical treatments warrant further investigation of antileishmanial compounds. *N*-myristoyltransferase has been demonstrated as a promising target for development of drugs treating leishmaniasis, particularly visceral leishmaniasis. Unfortunately, the current body of research on NMT-specific *leishmania* inhibitors is limited. In this case, existing target-free screening techniques may be easily combined to include screening for NMT inhibitors. The body of research on NMT inhibition in similar parasite and fungal species is thorough, with detailed SAR and crystallographic supporting evidence of binding modes on highly similar molecular targets. This offers an excellent starting point for piggyback development of *Leishmania*-specific NMT inhibitors. Of the few specific NMT inhibitors of *L. donovani* shown, no optimization has been performed on the parent chemical scaffolds, offering an opportunity to determine efficient syntheses, and relevant SAR for these compounds. Finally, *in silico* methods may be applied through out, in tandem with enzymatic assay results. In this manner, more efficient drug discovery methods may be accomplished, regardless of initial development method. The currently limited state of NMT-inhibitor development has been discussed, within the context of more expansive methods, applications, and relevant studies. Although there is a noticeable gap between these similar areas of research, several solutions have been proposed to further study of NMT inhibition of *L. donovani.*

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