Structure-Based Design of Potent and Selective *Leishmania* N-Myristoyltransferase Inhibitors

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*Supporting Information

**ABSTRACT:** Inhibitors of *Leishmania* N-myristoyltransferase (NMT), a potential target for the treatment of leishmaniasis, obtained from a high-throughput screen, were resynthesized to validate activity. Crystal structures bound to *Leishmania major* NMT were obtained, and the active diastereoisomer of one of the inhibitors was identified. On the basis of structural insights, enzyme inhibition was increased 40-fold through hybridization of two distinct binding modes, resulting in novel, highly potent *Leishmania donovani* NMT inhibitors with good selectivity over the human enzyme.

**INTRODUCTION**

The leishmaniases are a spectrum of infectious diseases caused by protozoan parasites of the genus *Leishmania*. Cutaneous leishmaniasis (CL), caused mainly by *Leishmania major* (Lm), can lead to permanent scarring and disfiguration, while visceral leishmaniasis (VL), caused mainly by *Leishmania donovani* (Ld), is often fatal due to failure of the host immune system. The leishmaniases are endemic in 88 countries, 72 of which are low-income, and is a major health issue with an estimated 0.2−0.4 million cases of VL, 0.7−1.2 million cases of CL, and a conservative estimate of 20000−40000 deaths per year. Treatment of leishmaniasis has previously been dominated by the use of pentavalent antimonials which are toxic, painful to administer, and require long treatment regimens; resistance has also developed to these antimonials in India. Some progress has been made in the last 10 years in the development of safer, more easily applied therapeutics with the development of lipid formulations of amphotericin B, miltefosine, and paromomycin. However, side effects are common and resistance to these therapies may still be a problem, thus the need for new antileishmanials remains high. Despite these issues, development of new antileishmanial drugs is limited and compounded by challenges of cell permeability. The amastigote form of the parasite most relevant to human disease resides within an acidic parasitophorous vacuole inside host cells, and the parasite bears a glycoinositolphospholipid coat that could limit uptake of xenobiotics.

N-Myristoyltransferase (NMT), an enzyme ubiquitous in eukaryotes, catalyzes the transfer of myristate (a 14-carbon fatty acid) to the N-terminal glycine of target proteins, either cotranslationally or post-translationally. Between 0.5% and 3% of the cellular proteome is predicted to be N-myristoylated, and this modification is vital for multiple regulatory processes, including protein−protein interactions and protein stability. Inhibition of NMT therefore has pleiotropic effects on cellular function. NMT has been shown to be essential in a range of parasitic organisms including *Leishmania*, and small-molecule cytotoxic inhibitors have been developed for NMTs in parasitic organisms including *Trypanosoma brucei* and *Plasmodium* species. Inhibition of *Leishmania* NMT therefore represents a rational drug target for development of new therapeutics for this neglected tropical disease.

The NMT enzyme operates via a Bi−Bi mechanism, with myristoyl CoA (MyrCoA) binding to the enzyme first and
inducing a conformational change before binding of the peptide substrate. The myristoyl group is then transferred to the N-terminal glycine of the peptide before sequential release of the myristoyl peptide and reduced CoA products. The structures of several parasitic NMTs have been reported and show a conserved binding site for MyrCoA. The peptide-binding region is less conserved between different species and therefore presents a target for selective inhibition of NMTs from different species.

A recently published high-throughput screen (HTS) of a diverse subset of the Pfizer corporate collection against LdNMT, Plasmodium falciparum NMT, and the two human isoforms (HsNMT1 and HsNMT2) revealed four novel series of Leishmania-selective NMT inhibitors. Here we report the development of highly potent LdNMT inhibitors based on structure-guided fusion of two of these series; piperidinylindoles, exemplified by PF-03393842, and aminoacylpyrrolidines, exemplified by PF-03402623 and PF-03402619 (Chart 1).

**RESULTS AND DISCUSSION**

Synthesis and Validation of Hits. To validate the HTS results, synthesis of both piperidinylindole 1 and the most potent aminoacylpyrrolidine 2 was carried out. Synthesis of 1 was achieved in four steps from 5-nitro indole (Scheme 1). Condensation of 5-nitro indole 4 with N-Boc-4-piperidone, followed by concurrent reduction of the resulting double bond and nitro group, yielded amine 6. Reaction with para-fluorophenylacetyl chloride followed by Boc deprotection gave piperidinylindole 1.

Compound 2 was synthesized as a mixture of two diastereoisomers, as it was unclear from the original report whether the stereochemistry at the pyrrolidine ring in the HTS hit was relative or absolute. It was envisaged that the preferred stereochemistry could be identified by cocrystallization of the mixture with Leishmania NMT. The pyrrolidine core was accessed as a racemic mixture by cycloaddition of benzyl(methyoxymethyl)(trimethylsilyl)methylamine 9 and trans-methyl 4-chlorocinnamate 8 in the presence of catalytic TFA to give trans-pyrrolidine 10 (Scheme 2). The benzyl protecting group was removed using 1-chloroethyl chlorofor-}

dyes, exemplified by PF-03393842, and aminoacylpyrrolidines, exemplified by PF-03402623 and PF-03402619 (Chart 1).

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**Table 1. Enzyme Activity Data (Results from HTS in Brackets)**

| compd | LdNMT IC\textsubscript{50} (μM) | LmNMT IC\textsubscript{50} (μM) | HsNMT1 IC\textsubscript{50} (μM) | EC\textsubscript{50} a (μM) | LD\textsubscript{50} b (μM) |
|-------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 1     | 0.31 (0.102)    | 0.55            | 63 (73)         | >30             | >45             |
| 2     | 0.080 (0.093)   | 0.031           | 4.7 (5.2)       | 10–30           | 8–16            |

EC\textsubscript{50} in extracellular Ld amastigotes; LD\textsubscript{50} in bone marrow derived mouse macrophages

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"Reagents and conditions: (a) pyrrolidine, N-Boc-4-piperidone, EtOH, rt, 3 days, 80%; (b) NH\textsubscript{4}HCO\textsubstitute{2}, EtOH, Pd/C, 2 h, 96%; (c) para-fluorophenylacetyl chloride, Et\textsubstitute{3}N, THF, 2 h, 92%; (d) 6 M HCl, IPA, 2 h, 43%.

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Chart 1. Leishmania-Selective Hits from Screening of a Subset of the Pfizer Compound File

![Chart 1](image-url)
Compounds were also tested against extracellular amastigotes of *Leishmania donovani* and against bone marrow derived macrophages to determine toxicity (Table 1). Compound 1 displayed no cell activity up to 30 μM, although no toxicity was observed. Compound 2 showed an EC<sub>50</sub> between 10 and 30 μM, however, the compound was also toxic to macrophages at this concentration.

**X-ray Crystallography.** Our first strategy to optimize these NMT inhibitors was to drive down enzyme potency using structure-guided design. To elucidate the binding mode of the HTS hits and the preferred stereochemistry of 2, crystal structures of ternary complexes of LmNMT (97% sequence homology with LdNMT) and myristoyl-CoA cofactor were obtained for both resynthesized hits, as recently reported. Both inhibitors were shown to bind in the peptide binding region. The structure of compound 1 bound to LmNMT revealed a direct interaction between the basic piperidine nitrogen and the C-terminal carboxylate of the enzyme (Leu421) (Figure 1). This type of charge−charge interaction has previously been observed with other NMT inhibitors in *Plasmodium* NMT<sup>20,21</sup> and via a bridging water molecule in LmNMT.<sup>19</sup> The indole adopts an equatorial position off the piperidine ring in a hydrophobic pocket, and the amide carbonyl is orthogonal to the indole ring, forming hydrogen bonds to Tyr345 and Asn376.

The cocrystal structure of 2 bound to LmNMT displays a unique binding mode compared to previously reported NMT inhibitors; the conformation of the inhibitor appears to be governed by a hydrophobic collapse<sup>38</sup> that folds the aromatic rings into a hairpin conformation about the flexible linker, with the chlorophenyl substituent of the pyrrolidine ring sandwiched between the edge of Tyr345 below and Tyr217 above. The inhibitor takes up a compact conformation in which its surface area is almost completely buried by the protein and MyrCoA. Interestingly, the key charge−charge interaction between the basic amine and Leu421 is not seen (Figure 2). Instead, the primary amine is adjacent to the thioester of MyrCoA and makes bridging contacts with the backbone carbonyl of Thr203 and the side chain of Asn167. The hydroxyl group is actually closest to the C-terminal leucine carboxylate (2.6 Å), and there is a potential hydrogen bond between the amide carbonyl and Thr203 (Figure 2).

As expected, the crystal structure shows a single diasteroisomer (2a, Scheme 3) bound to the enzyme. To confirm that 2a is the most active isomer, both diastereoisomers (2a and 2b) were synthesized separately using enantiopure oxazolidinone 17 for the cycloaddition reaction (Scheme 3).

Cycloaddition yielded diasteroisomers 18a and 18b, which could be separated by column chromatography and were assigned by comparison with reported 1H NMR data<sup>39</sup> (Scheme 3). Removal of the oxazolidinone gave esters 10a and 10b from which 2a and 2b were synthesized, respectively, using the route detailed in Scheme 2.

Enzyme inhibition assays confirmed that diastereoisomer 2a was more active with an IC<sub>50</sub> of 25 nM against LdNMT, with 2b exhibiting 60-fold lower potency and 4-fold lower selectivity for LdNMT over HsNMT<sub>1</sub> (Table 2).

A crystal structure was also obtained for 2b bound to LmNMT. The structure shows a similar hydrophobic collapse of the ligand and that the key functional groups (the primary

**Table 2. Enzyme Activity Data for Diastereoisomers 2a and 2b**

| compd | LdNMT IC<sub>50</sub> (μM) | HsNMT<sub>1</sub> IC<sub>50</sub> (μM) |
|-------|-----------------|-----------------|
| 2     | 0.080           | 4.7             |
| 2a    | 0.025           | 1.4             |
| 2b    | 1.7             | 24              |
amine and alcohol) of both diastereoisomers are superimposed in the active site (circled, Figure 3). However, as a result of maintaining these interactions, the scaffold is twisted such that the amide carbonyl no longer forms the hydrogen bond with Thr203 seen in the structure of 2a.

**Hybridization of Binding Modes.** Comparison of the distinct binding modes of hits 1 and 2 showed that the benzoo-ring of the indole in 1 and the aromatic substituent of the pyrrolidine in 2 bind in the same region (Figure 4a). For this reason, it was hypothesized that addition of a para-fluorophenyl acetamide ortho- to the chlorine atom in this ring in compound 2 may significantly improve potency (Figure 4b). This could potentially introduce hydrogen bonding between the acetamide carbonyl and Tyr345 and Asn376 and allow the same hydrophobic pocket as the amide carbonyl no longer forms the hydrogen bond with Thr203 seen in the structure of 2a.

**Scheme 4. Synthesis of 19 and 20**

![Scheme 4](image)

Reagents and conditions: (a) 9, TFA, DCM, 0 °C to rt, 24 h, 79%; (b) SnCl₂, EtOH, 2 h, 87% (c) R = H, Ac₂O, Et₃N, DCM, 2 h, 65%, R = p-F-Ph para-fluorophenylacetyl chloride, Et₂N, DCM, 2 h, 42%; (d) (i) 1-chloroethyl chloroformate, toluene, 110 °C, 3 h, (ii) MeOH, reflux, 30 min; (e) EDCI, HOBT, DIPEA, 12, DMP, 4 h, R = H 41% over 2 steps, R = p-F-Ph 43% over 2 steps; (f) LiBH₄, THF, 3 h; (g) TFA, DCM, 2 h, R = H 61% over 2 steps, R = p-F-Ph 34% over two steps.

X-ray crystal structures of 20, 1, and 2 bound to LmNMT demonstrates that 20 binds as designed and that all interactions with the enzyme are conserved (Figure 5 and Figure S1, Supporting Information).

**Table 3. Enzyme and Cell Activity Data**

| compd | pKᵢ | LD₅₀ (nM) | IC₅₀ (nM) | EC₅₀ (μM) | LD₅₀ (μM) |
|-------|-----|----------|----------|-----------|-----------|
| 1     | 10.0| 254      | 28505    | >30       | >45       |
| 2     | 8.9 | 63       | 2124     | 10–30     | 8–16      |
| 2a    | 8.9 | 17       | 631      | 10–30     | 12–24     |
| 2b    | 8.9 | 1406     | 10857    | 10–30     | 12–24     |
| 19    | 8.9 | 110      | 4910     | >50       | >90       |
| 20    | 8.9 | 1.6      | 27       | 10–30     | 12–24     |
| 43    | 59  | 1710     |          | 10–30     | >24       |

EC₅₀ in extracellular Ld amastigotes (for comparison, EC₅₀ for the widely used antileishmanial drugs amphotericin B and miltefosine in this assay are 50 and 7850 nM, respectively.) LD₅₀ in bone marrow-derived mouse macrophages.
based activity was seen (Table 3). This highlights that simply driving down enzyme potency in this compound series is insufficient to increase cellular activity against this challenging target organism. The diastereoisomers of 2 were also tested separately, and both displayed an EC\textsubscript{50} of 10–30 \textmu M and an LD\textsubscript{50} of 12–24 \textmu M, demonstrating that both activity and toxicity are unrelated to NMT inhibition for these compounds. We hypothesized that the lack of cell-based activity for this series of compounds is due to lack of cellular uptake and thus, insufficient target engagement. Compounds 1 and 2 and derivatives synthesized here all contain a basic center (pK\textsubscript{a} 10.0 and 8.9 respectively) which would be charged at physiological pH, with a potentially adverse effect on membrane permeability.

Replacement of the Primary Amine. As the crystal structures of the aminoacylpyrrolidines show that the amine does not make the key charge–charge interaction with the C-terminal carboxylate observed previously in other series, we considered replacing the primary amine with a less basic moiety. It was envisaged that the amine could be replaced with an alcohol without loss of hydrogen bonding, potentially generating a potent, neutral NMT inhibitor. To synthesize this neutral compound, acid 35 was synthesized from 4-chlorophenylacetyl chloride 32. Reaction with Meldrum’s acid followed by hydrolysis gave ketone 33, which was subsequently reduced. Hydrolysis of the resulting ester gave the acid 35 (Scheme 5).

**Scheme 5. Synthesis of Acid 35\textsuperscript{a}**

\[ \text{Scheme 5. Synthesis of Acid 35} \]

\textsuperscript{a}Reagents and conditions: (a) (i) Meldrum’s acid, pyridine, DCM, 0 °C, 30 min then rt, 15 h, (ii) EtOH, reflux, 2 h, 70%; (b) NaBH\textsubscript{4}, MeOH, 0 °C to rt, 1.5 h, 40%; (c) LiOH, MeOH/H\textsubscript{2}O 99%.

This acid was then used to synthesize the alcohol analogue of hybrid 20 (Scheme 6). As acid 35 was synthesized as a racemic mixture, pyrrolidine 39 was synthesized as a single enantiomer in order to give the hybrid alcohol 43 as a mixture of only two diastereoisomers (Scheme 6). The hybrid alcohol 43 was tested for its enzyme activity and showed reduced activity compared to the corresponding amine (Table 3). However, the activity for this neutral compound is comparable to the original primary amine hit 2, and selectivity over HsNMT1 is maintained.

The crystal structure of alcohol 43 bound to LmNMT appeared to overlay well with that of amine 20 (Figure S2, Supporting Information, PDB code 4cyq). However, closer inspection revealed a slight difference in the position of the amine versus the alcohol in these two structures. The primary amine of 20 forms a hydrogen bond to the backbone carbonyl group of Thr203 at a distance of 2.9 Å. When this amine is replaced with an alcohol in compound 43, the corresponding oxygen is 3.5 Å away, reducing its potential to hydrogen bond.

Testing against extracellular amastigotes showed no improvement in activity for compound 43 compared with the original hits or the hybrid amine (Table 3). Metabolic chemical tagging\textsuperscript{22} in Ld amastigotes (Figure S3, Supporting Information) demonstrated that despite replacement of the primary amine, target engagement consistent with a K\textsubscript{i} of 59 nM was not achieved, supporting our hypothesis that the lack of cell-based activity for these compounds is due to poor cellular uptake.

Despite the potential of NMT as a drug target in Leishmania, these organisms are known to be difficult to target, due in part to their cell surface coats, a key component of Leishmania virulence and survival.\textsuperscript{9,10} Advances in potency and particularly physicochemical properties will be required to progress this series of compounds and to chemically validate Leishmania NMT as a drug target in vivo.

**CONCLUSION**

Two Leishmania NMT-selective HTS hits have been resynthesized and their activities validated. Crystal structures of these inhibitors identified their binding modes and, in the case of compound 2, identified the active diastereoisomer. The crystal structures were used to increase enzyme affinity through hybridization of the two independent binding modes, and this led to the discovery of a highly potent inhibitor of LdNMT. The unusual binding mode of the aminoacylpyrrolidines allowed the replacement of the primary amine, leading to compound 43, a potent and neutral NMT inhibitor. Although poor uptake appears to lead to a lack of cell activity for these compounds, elucidation of the binding modes of these inhibitor series along with their hybridization provides a useful starting point for the development of LdNMT inhibitors with improved physicochemical properties.

**EXPERIMENTAL SECTION**

The purity of final compounds was determined by reversed-phase LC-MS on a Waters 2767 system and was >95% for all tested compounds. (+)-(3R,4S)-Methyl 1-Benzyl-4-(4-chlorophenyl)pyrrolidine-3-carboxylate (10). TFA (20 \textmu L, 0.20 mmol) was added to a solution of 9 (1.04 mL, 4.06 mmol) and chlorocinnamate 8 (400 mg,
2.03 mmol) in DCM (40 mL) at 0 °C, and the solution was stirred (rt) for 24 h. Saturated aqueous NaHCO₃ (40 mL) was added, and the phases were separated. The organic layer was dried over Na₂SO₄, and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (1:9 EtOAc–hexane, Rf 0.25) to give the product 10 as a colorless oil (590 mg, 88%).

1° H NMR (400 MHz, CDCl₃) δ 7.41–7.27 (m, 9H), 3.76–3.68 (m, 3H), 3.68–3.63 (m, 1H), 3.16–3.03 (m, 2H), 3.02–2.96 (m, 1H), 2.86 (dd, J = 8.3, 6.4 Hz, 1H), 2.77 (dd, J₁ = 9.4, 5.8 Hz, 1H).

(3R,4S) Methyl 1-(R)-3-((tert-Butyloxy)carbonyl)amo)no-4-(4-chlorophenyl)butanoyl)-4-(4-chlorophenyl)pyrrolidine-3-carboxylate (13). 1-Chloroethyl chloroformate (327 μL, 3.03 mmol) was added to a solution of 10 (500 mg, 1.51 mmol) in toluene (30 mL), and the solution was stirred at 110 °C for 3 h. The reaction was cooled to room temperature, and the solvent was removed under reduced pressure. The residue was dissolved in methanol (30 mL), and the phases were separated. The organic layer was dried over Na₂SO₄ and the solvent removed under reduced pressure. The crude residue was purified by column chromatography (1:1 EtOAc–hexane, Rf 0.35) to give the product 13 as a colorless oil (186 mg, 55%) as a mixture of diastereomers and as a mixture of amide rotamers by ¹H NMR at room temperature. ¹H NMR (400 MHz, CDCl₃) δ 7.38–7.24 (m, 4H), 7.23–7.08 (m, 4H), 5.68 (br s, 1H), 4.18–3.97 (m, 2H), 3.85–3.42 (m, 6H), 3.27–3.10 (m, 1H), 3.10–3.00 (m, 1H), 2.97–2.85 (m, 1H), 2.50–2.40 (m, 2H), 1.45–1.38 (m, 9H).

tert-Butyl (R)-1-(4-Chlorophenyl)-4-(((3S,4R)-3-(4-chlorophenyl)-4-(hydroxymethyl)pyrrolidin-1-yl)-4-oxobutan-2-yl)carbamate (14). LiOH (3 mg, 0.13 mmol) was added to a solution of 13 (18 mg, 0.04 mmol) in dry THF (1 mL). The solution was stirred for 3 h (rt). Water was added (2 mL), followed by DCM (2 mL), and the phases were separated. The organic layer was dried over MgSO₄ and the solvent removed under reduced pressure to give the product 14 as a colorless oil (12 mg, 71%) as a mixture of diastereomers and as a mixture of amide rotamers by ¹H NMR at room temperature. ¹H NMR (400 MHz, CDCl₃) δ 7.36–7.24 (m, 4H), 7.17 (m, 4H), 5.89–5.59 (br s, 1H), 4.18–3.86 (m, 2H), 3.77–3.63 (m, 2H), 3.61–3.09 (m, 4H), 3.04 (m, 1H), 2.95–2.84 (m, 1H), 2.54–2.38 (m, 2H), 1.46–1.36 (m, 9H).

(R)-3-Amino-4-(4-chlorophenyl)-1-(((3R,4R)-3-(4-chlorophenyl)-4-(hydroxymethyl)pyrrolidin-1-yl)butan-1-one (2). TFA (11 μL, 0.11 mmol) was added to a solution of 14 (12 mg, 0.02 mmol) in DCM (1 mL), and the reaction was stirred at 2 h (rt). The solvent was removed under reduced pressure, and the crude residue was purified by preparative LCMS (method B) to give the product 2 as a colorless oil (3 mg, 31%) as a mixture of diastereomers. ¹H NMR (400 MHz, MeOD) δ 7.43–7.24 (m, 8H), 4.06–3.76 (m, 2H), 3.72 (s, 1H), 3.61–3.55 (m, 1H), 3.52–3.37 (m, 3H), 3.29–3.14 (m, 1H), 2.99–2.85 (m, 2H), 2.72–2.39 (m, 3H). m/z 407 ([M + H]+). HRMS found 407.1313, C₂₃H₂₂N₂O₂Cl requires 407.1293. LCMS Rf = 12.44 min. Complete experimental details including LCMS methods are provided in the Supporting Information.

**ASSOCIATED CONTENT**

**Supporting Information**

Experimental procedures and characterization of all compounds, assay procedures, X-ray data collection and statistics, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

**Accession Codes**

The coordinates and structure factor files have been deposited in the Protein Data Bank with accession codes 4cgn (LmNMT MyrCoA-A1), 4cgl (LmNMT-MyrCoA-A2a), 4cyn (LmNMT-MyrCoA-A2b), 4cyo (LmNMT-MyrCoA-A20), and 4cyq (LmNMT-MyrCoA-A43).

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**Author Contributions**

All authors have given approval to the final manuscript.

**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS USED**

Ld, Leishmania donovani; Lm, Leishmania major; NMT, N-myristoyltransferase; Hs, Homo sapiens; CPM, 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin; MyrCoA, myristoyl-CoA.

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