Discovery of Novel and Ligand-Efficient Inhibitors of \textit{Plasmodium falciparum} and \textit{Plasmodium vivax} N-Myristoyltransferase

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\textbf{Supporting Information}

\textbf{ABSTRACT:} N-Myristoyltransferase (NMT) is an attractive antiprotozoan drug target. A lead-hopping approach was utilized in the design and synthesis of novel benzo[\(b\)]thiophene-containing inhibitors of \textit{Plasmodium falciparum} (Pf) and \textit{Plasmodium vivax} (Pv) NMT. These inhibitors are selective against \textit{Homo sapiens} NMT1 (HsNMT), have excellent ligand efficiency (LE), and display antiparasitic activity in vitro. The binding mode of this series was determined by crystallography and shows a novel binding mode for the benzothiophene ring.

\section{INTRODUCTION}

Diseases resulting from parasitic infections are a global health crisis, responsible for over 700,000 annual deaths and predominantly affecting developing countries.\textsuperscript{1} The most serious is malaria, caused by parasites of the genus \textit{Plasmodium}. The vast majority of malaria infections stem from the species \textit{Plasmodium falciparum} (Pf) and \textit{Plasmodium vivax} (Pv). Emerging resistance to current therapies highlights the urgent requirement for new antimalarial medications in the near future.\textsuperscript{2}

N-Myristoyltransferase (NMT) is an enzyme found exclusively in eukaryotes and is responsible for the co- and post-translational attachment of the C\(_{14}\) fatty acid myristic acid to the N-terminal glycine of substrate proteins.\textsuperscript{3} In malaria, essential proteins such as calcium dependent protein kinase 1\textsuperscript{4} and glideosome associated protein 4\textsuperscript{5} have been shown to require myristoylation to carry out their biological functions. Furthermore, genetic experiments have shown NMT to be essential in \textit{Plasmodium berghei} in vivo.\textsuperscript{6} This evidence strongly suggests that NMT is a highly promising antiparasitic drug target.

\section{RESULTS AND DISCUSSION}

Our previous work has led to the identification of parasite NMT inhibitors via high throughput screening.\textsuperscript{7,8} As an alternative strategy for hit discovery, NMT has been highlighted as a target for the piggy-back approach.\textsuperscript{9} We have used this methodology successfully to produce a series of moderate affinity and selective PfNMT inhibitors adapted from antifungal NMT inhibitors developed initially by Roche (1, Table 1).\textsuperscript{10} Although 1 displays selectivity over the human NMT orthologues (HsNMT) and moderate enzyme affinity, its relatively large size means that the ligand efficiency (LE) is significantly lower than 0.35, the average LE of high-throughput screening hits.\textsuperscript{11} A poor LE limits the potential of a series in hit to lead development, increasing the chances of later stage attrition. We therefore sought to develop this series with the aim of producing more ligand efficient, selective, and novel hit series for PfNMT and PvNMT.

On the basis of the available crystallographic information,\textsuperscript{12} it was hypothesized that lead hopping by moving the amine substituent from the 4-position on the benzo[\(b\)]furan scaffold to the 3-position would be tolerated by the enzyme. This modification would allow the exploration of novel chemical space, facilitating the discovery of novel parasitic NMT inhibitors.

Synthesis of the template was achieved by a Williamson ether synthesis followed by Dieckmann condensation, affording 3 in high yield. A Mitsunobu reaction and deprotection resulted in 5, designed as an analogue of 1 (Scheme 1). Pleasingly, this shift in substitution pattern resulted in a 10-fold affinity improvement against PfNMT, a 3-fold improvement against PfNMT, and no measurable activity against HsNMT up to 100 \(\mu\)M. Coupled with the loss of one heavy atom, this improved the LE to 0.38 for PfNMT and 0.35 for PfNMT (Table 1).

A range of amines was synthesized to investigate the linker length, basicity, and lipophilicity requirements (see Supporting Information). All changes resulted in complete loss of affinity against all three enzymes, reinforcing previous results from a related series that the piperidine substituent is strongly preferred for affinity.\textsuperscript{10} For each of these syntheses, the Mitsunobu reaction proceeded with a disappointingly poor yield, typically less than 50%. It was hypothesized that despite the potential for an intramolecular hydrogen bond, the weakly aromatic furan ring\textsuperscript{13} resulted in significant tautomerism between 3 and the undesired ketone tautomer 6 (Figure 1). Mitsunobu reactions utilizing the disfavored enol tautomer have been previously reported with reactive electrophiles;\textsuperscript{14} however, it is also known that the presence of carbonyls\textsuperscript{15} and...
highly acidic α-carbonyl protons\textsuperscript{16} can produce side reactions. Furthermore, the unstable heterocycle may lead to problems with stability and toxicity due to the presence of an (albeit hindered) α,β-unsaturated carbonyl in 5 not stabilized by aromaticity.

It is known that the aromatic stabilization energy of benzo[b]thiophenes is far greater than that of benzo[b]furans\textsuperscript{13} and that this results in a higher population of the enol tautomer.\textsuperscript{17} It was therefore proposed that the bioisosteric replacement of the benzo[b]furan core with benzo[b]thiophene may improve the synthetic efficiency. Furthermore, the benzo[b]furan scaffold is known to form π-interactions with Tyr334 and Ty211 in PvNMT;\textsuperscript{10} therefore, the increased aromatic character of the benzo[b]thiophene scaffold may result in improved interactions between the scaffold and these residues.

Replacement of oxygen with sulfur enabled modification of the Williamson ether synthesis/Dieckmann condensation into a one-pot cascade procedure and dramatically improved the yield of the Mitsunobu ether synthesis from 26% to 97% (Scheme 2).

Scheme 1. Synthesis of Benzo[b]furan 1\textsuperscript{a}

\begin{equation}
\begin{align*}
\text{Scheme 2. Synthesis of Benzo[b]thiophene 9}\textsuperscript{a} & \\
\end{align*}
\end{equation}

\textsuperscript{a}Reagents and conditions: (a) ethyl bromoacetate, K\textsubscript{2}CO\textsubscript{3}, acetone, reflux, 3 h, 96%; (b) t-BuOK, THF, rt, 15 min, 94%; (c) 1-Boc-4-piperidinol, disopropyl azodicarboxylate, PPh\textsubscript{3}, THF, rt, 18 h, 26%; (d) 10% TFA in DCM (v/v), rt, 2 h, 97%.

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Scheme 2. Synthesis of Benzo[b]thiophene 9\textsuperscript{a}

\begin{equation}
\begin{align*}
\end{align*}
\end{equation}

\textsuperscript{a}Reagents and conditions: (a) ethyl bromoacetate, t-BuOK, THF, rt, 15 min, 88%; (b) 1-Boc-4-piperidinol, disopropyl azodicarboxylate, PPh\textsubscript{3}, THF, rt, 1.5 h, 97%; (c) 10% TFA in DCM (v/v), rt, 2 h, 82%.
Although incorporation of 3-methoxyphenyl (12b, Table 1) produced only a modest improvement in PnPMT enzyme affinity (9 vs 12b, Table 1), the improvement in PvNMT is far more pronounced. In addition, 12b is the most potent antiplasmodial compound of this series with an EC₅₀ of 2.0 μM.

The results in Table 1 indicate that the targeted interactions from the additional aromatic group are not being formed; the benzyl ester 12a is less potent than 9, implying suboptimal interactions for this substituent. Nonetheless, 12b displays excellent PvNMT enzyme affinity and is 10-fold selective over PnPMT. This is in contrast to previous results with a related series, indicating the potential for a novel binding mode of this series compared to the 2,3,4-benzofuran analogues (exemplified by 1).^{10}

The strong enzyme affinity of 12b for PvNMT facilitated determination of the structure of 12b bound to the active site of PnPMT (Figure 2, PDB accession code 4BBH). As implied by the structure–activity relationship in Table 1, 12b adopts an overlapping but distinct binding mode to the 2,3,4-benzofuran series (Figure 2C, PDB accession code 4B14). The interaction between the basic amine moiety and the α-carboxylate of the C-terminal leucine of the protein appears to dominate (Figure 2A), with both inhibitors aligning to form this interaction (Figure 2C). The chemically similar benzofuran and benzothiophene rings occupy distinct locations within the enzyme pocket (Figure 2C). The altered substitution pattern results in the benzothiophene scaffold being buried deeper within a hydrophobic pocket (Figure 2B), providing a rationalization for the high PvNMT enzyme affinity of 12b. As a result of the displacement of the scaffold, the methoxyphenyl group of 12b is unable to reach the pocket occupied by this substituent in the 2,3,4-benzofuran inhibitor (Figure 2C). This results in suboptimal binding of this portion of the molecule, with multiple alternative binding positions visible in the three protein chains present in the asymmetric unit (Figure 2B,C). There is clearly scope for optimizing this substituent, perhaps by extending the linker between the methoxyphenyl group and the scaffold to reach the targeted pocket.

The fluctuating selectivity of inhibitors in this series for NMT orthologues is difficult to rationalize based on the crystallographic information; the only residue difference within 5 Å of the active site is the substitution of Tyr334 in PvNMT

Throughout this series, amides displayed consistently lower affinity than esters, and the benzyl substituent resulted in the loss of a great deal of selectivity compared to ethyl ester 9.

**Figure 2.** X-ray structure of inhibitors bound to PvNMT.^{18} (A) A crucial interaction between the enzyme and inhibitor involves the amine of the piperidine moiety of 12b (purple) and a hydrophilic pocket incorporating the enzyme C-terminal carboxylate (Leu10). (B) In this binding mode the benzyl thiophene scaffold is deeply buried within the binding pocket. Although this portion of the inhibitor is well-defined, the electron density identifies multiple alternative binding positions for the benzyl ester moiety, as shown by thin bonds (see Supporting Information). This substituent is clearly suboptimal, suggesting an area of future development for this series. (C) The contrasting binding modes are clearly shown by overlay of the PvNMT complex formed by 12b (purple) with that formed by a previously discovered 2,3,4-benzofuran inhibitor (green).^{10} The novel binding mode explains the difference in SAR between the two series.
by Phe334 in PfNMT, with HsNMT and PrNMT displaying a completely homologous active site. The reasons for selectivity are likely to be more subtle, perhaps because of long-range factors modulating stereoelectronic interactions or protein dynamics in the binding site.

The current series contains a potentially biologically labile ester group, and as yet we have no information about the metabolic stability. Further development will focus on compound stability with potency and selectivity to produce a robust lead series for further development.

**CONCLUSION**

Analysis of structural information indicated the potential for chemical diversity of a Pf/PvNMT inhibitor via a lead-hopping approach. Altering the positions of scaffold substitution and scaffold-hopping based on aromatic stabilization yielded highly selective and ligand efficient 9 that is built around a benzo[b]thiophene core. Attempts to translate structure–activity relationships developed in a related series yielded 12b. Compound 12b is a structurally novel, high affinity PfNMT inhibitor that displays excellent LE and antiplasmodial activity in vitro. The crystal structure of 12b bound to PfNMT highlighted a novel binding mode for this scaffold.

**EXPERIMENTAL SECTION**

Purity of tested compounds was ≥95% unless otherwise specified, as confirmed by LC–MS.

**Ethyl 3-Hydroxybenzo[b]thiophene-2-carboxylate 7.** To a solution of methyl 2-mercaptobenzoate (1.63 mL, 12.2 mmol) in THF (130 mL) at 0 °C was added potassium tert-butoxide (5.14 g, 71.3 mmol) gradually over 2 min. The mixture was stirred and allowed to warm to rt over 15 min, quenched with 2 M HCl solution to pH 2, and diluted with 75 mL of water. 7 was extracted with 3 × 75 mL portions of EtOAc. The organic layers were combined, washed with 75 mL of brine, dried over MgSO4, and concentrated under reduced pressure. The crude product was purified by flash chromatography, yielding 11b as a colorless oil (37 mg, 26%). 1H NMR (CDCl3, δ ppm) 7.86 (1H, d, J = 8.0), 7.75 (1H, d, J = 8.2), 7.51–7.46 (1H, m), 7.43–7.37 (1H, m), 7.32 (1H, apparent t, J = 7.9), 7.04 (1H, d, J = 7.8), 7.02–7.00 (1H, m), 6.90 (1H, dd, J = 8.2, 2.3), 5.35 (2H, s), 4.74–4.66 (1H, m), 3.94–3.86 (2H, m), 3.84 (3H, s), 3.07–2.98 (2H, m), 1.98–1.88 (2H, m), 1.85–1.73 (2H, m), 1.48 (9H, s).

**3-Methoxybenzyl-3-(piperidin-4-yl)benzo[b]thiophene-2-carboxylate 12b.** To a solution of 11b (34 mg, 0.07 mmol) in DCM (1.00 mL) was added TFA (100 μL). The solution was stirred at rt for 2 h. The mixture was concentrated under reduced pressure and purified by HPLC, yielding 12b as a colorless oil (5 mg, 18%). tR = 12.3 min; 1H NMR (CDCl3, δ ppm) 7.82 (1H, d, J = 8.0), 7.76 (1H, d, J = 8.1), 7.56–7.48 (1H, m), 7.47–7.40 (1H, m), 3.73 (1H, apparent t, J = 7.9), 7.04 (1H, d, J = 7.4), 7.01–6.98 (1H, m), 6.91 (1H, dd, J = 8.2, 2.4), 5.34 (2H, s), 4.88–4.81 (1H, m), 3.84 (3H, s), 3.54–3.44 (2H, m), 3.11–3.01 (2H, m), 2.22–2.11 (4H, m). 13C NMR (CDCl3, δ ppm) 161.48, 159.84, 153.90, 138.23, 136.98, 134.06, 129.82, 128.39, 125.07, 123.14, 123.61, 120.34, 115.87, 113.81, 113.79, 76.10, 66.82, 55.29, 41.01, 28.07. ESI HRMS, found 398.1425 [C16H13NO5S [M + H]+], requires 398.1426.

**ASSOCIATED CONTENT**

**Supporting Information**

Experimental procedure, characterization of intermediates and target compounds, description of biological assays, determination of K values, biological data of supplementary compounds, and crystallographic information. 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 under the accession code 4BBH.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS USED**

nd, not determined; Pf, Plasmodium falciparum; Pv, Plasmodium vivax; NMT, N-myristoyltransferase; Hs, Homo sapiens
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(18) This figure was produced using the program PyMol (www.pymol.org). The protein surfaces are color-coded according to the electrostatic surface potentials: red is negatively charged; blue is positively charged.