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N-Myristoyltransferase as a potential drug target in malaria and leishmaniasis

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1 *Title: N*-Myristoyltransferase as a potential drug target in malaria and leishmaniasis

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11 *Leishmania donovani*, *Plasmodium falciparum*, high throughput screening

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12 SUMMARY

13

14 Infections caused by protozoan parasites are among the most widespread and intractable transmissible
15 diseases affecting the developing world, with malaria and leishmaniasis being most costly in terms of
16 morbidity and mortality. Although new drugs are urgently required against both diseases in the face of
17 ever-rising resistance to frontline therapies, very few candidates passing through development
18 pipelines possess a known and novel mode of action. Set in the context of drugs currently in use and
19 under development, we present the evidence for *N*-myristoyltransferase (NMT), an enzyme that *N*-
20 terminally lipidates a wide range of specific target proteins through post-translational modification, as
21 a potential drug target in malaria and the leishmaniases. We discuss the limitations of current
22 knowledge regarding the downstream targets of this enzyme in protozoa, and our recent progress
23 towards potent cell-active NMT inhibitors against the most clinically-relevant species of parasite.
24 Finally, we outline the next steps required in terms of both tools to understand *N*-myristoylation in
25 protozoan parasites, and the generation of potential development candidates based on the output of
26 our recently-reported high-throughput screens.

27 INTRODUCTION

28 *Malaria*

29 Malaria is a disease caused by infection of a human host with protozoan parasites of the genus
30 *Plasmodium*, and is a devastating global health issue with approximately 200 million cases and
31 1 million deaths in 2010 alone (Murray *et al.* 2012). The complex life cycle of malaria parasites
32 spreads across two hosts and five host tissues whilst undergoing at least ten distinct morphological
33 transitions (Sturm *et al.* 2006; Mackinnon and Marsh 2010). Replication of parasites and subsequent
34 rupture of erythrocytes in the intra-erythrocytic stages are responsible for the clinical symptoms of
35 malaria, and the majority of drugs target these asexual (human-host) stages of the life cycle. Some
36 species of malaria, most notably *Plasmodium vivax,* can exist in a latent liver hypnozoite form that
37 can cause relapse even after clearance of bloodstream parasites (Derbyshire *et al.* 2012; Rodrigues *et*

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38 *al.* 2012). Of the five relevant species of human parasite, the vast majority of deaths occur from *P.*
39 *falciparum* infections, which is the typical cause of severe malaria (Claessens *et al.* 2012). This has
40 led to the majority of drug discovery efforts focussing on *P. falciparum*, typically at the expense of
41 other species. Although the demand for new *P. falciparum* drugs is in no doubt, *P. vivax* is
42 responsible for the majority of worldwide malaria endemicity (Price *et al.* 2009; WHO 2011).
43 However, difficulties culturing the parasite (Udomsangpetch *et al.* 2007) along with challenges
44 imaging and targeting the hypnozoite liver stages (Meister *et al.* 2011) have led to a dearth of new *P.*
45 *vivax* drugs (Price *et al.* 2009). Medications capable of targeting all relevant species of parasite, and
46 crucially clearing liver-stage parasites, are in great demand.

47 For the latter half of the 20th century, antimalarial drug discovery was a success story for natural
48 product-inspired therapies, by far the most widely used of which are chloroquine (Loeb *et al.* 1946)
49 and artemisinin (Miller and Su 2011).

50 Chloroquine was first discovered as a derivative of antimalarial natural product quinine (Krafts *et al.*
51 2012), and has been a first-line antimalarial for over sixty years (Loeb *et al.* 1946). Studies over the
52 past twenty years have shown that this class of compounds (with the exception of primaquine) is
53 involved in the disruption of haem detoxification by the parasite (Weissbuch and Leiserowitz 2008).
54 Artemisinin is a highly effective antimalarial natural product, isolated from *Artemisia annua* (Miller
55 and Su 2011); its antimalarial action is still under debate, but most hypotheses involve reductive
56 activation of the endoperoxide moiety resulting in parasite death from oxidative damage (O'Neill and
57 Posner 2004; Li and Zhou 2010; Slack *et al.* 2012). Other drug classes used in the treatment of
58 malaria to varying extents include pyrimidine biosynthesis disruptors, drugs that target the apicoplast
59 (Botté *et al.* 2012) and drugs discovered by phenotypic screening with unknown targets (e.g.
60 primaquine) (Kappe *et al.* 2010). Despite the apparent plethora of antimalarials, drug resistance is a
61 major issue and new medications with distinct mechanisms are constantly required to combat the
62 continued evolution of the parasite (Fidock 2010; Mackinnon and Marsh 2010). This is compounded
63 by the emergence of resistance to the artemisinins in Asia, (Dondorp *et al.* 2009; Phyo *et al.* 2012)
64 reinforcing the urgent requirement for new therapies. Fortunately, a great deal of resource has been

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65 directed towards antimalarial drug discovery in the past few decades. Elaboration of the artemisinin
66 pharmacophore has resulted in multiple clinical candidates (Vennerstrom *et al.* 2004; O'Neill *et al.*
67 2010; Charman *et al.* 2011), although the potential effectiveness of these compounds in artemisinin-
68 resistant regions remains a concern due to the shared mechanism of action. In addition, novel
69 inhibitors of the pyrimidine biosynthetic pathway are in development (Painter *et al.* 2007) and
70 phenotypic high-throughput screens have resulted in a wealth of information on relevant scaffolds
71 (Plouffe *et al.* 2008; Gamo *et al.* 2010; Guiguemde *et al.* 2010), and yielded promising clinical
72 candidates (Rottmann *et al.* 2010). Among the numerous clinical and preclinical candidates for the
73 treatment for malaria, the vast majority work by existing or unknown mechanisms and are based on
74 known pharmacophores; indeed many are new combinations of existing marketed drugs. Although
75 some have shown efficacy against resistant strains, concerns remain that resistance may develop
76 quickly against already vulnerable mechanisms. New drugs that work by distinct novel biological
77 mechanisms are therefore highly desirable.

78 *The leishmaniases*

79 The leishmaniases are the second most prevalent class of parasitic infection after malaria, giving rise
80 to >2 million new cases each year. The disease occurs in three forms, cutaneous (CL), muco-
81 cutaneous (MCL) and the most fatal form, visceral leishmaniasis (VL). The latter is associated with
82 infection by the species *Leishmania donovani,* while the cutaneous forms are due to infection by
83 multiple species including *L. major*, *L. braziliensis* and *L. mexicana*. The leishmaniases are endemic
84 in more than 90 countries around the world, being particularly prevalent in India, East Africa,
85 Bangladesh and Brazil. Additional clinical issues include post-kala-azar dermal leishmaniasis
86 (PKDL), occurring after the apparent drug cure of VL in certain geographical regions (e.g. Sudan) and
87 difficult to cure with pentavalent antimonials. Combination therapies with therapeutic vaccines
88 (Maroof *et al.* 2012) or immune-response activating drugs, such as imiquimod (Arevalo *et al.* 2001),
89 show some promise. In contrast to the wealth of treatments and drugs in development for malaria, the
90 leishmaniases are poorly provided for. None of the currently available drugs (Fig. 2) were discovered
91 by a rational design process for this neglected disease, and suffer from drawbacks including lack of an

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92 oral formulation, prolonged treatment times, high cost of treatment, toxicity, teratogenicity and/or
93 increasing drug resistance. In addition, all work through unknown mechanisms, by disruption of cell
94 membranes or through unspecific antibiotic effects. There are very few drugs in development for
95 these conditions with the most advanced being another antibiotic, fexinidazole (Winkelmann and
96 Raether 1978), discovered as part of a repurposing initiative (Wyllie *et al.* 2012).

97 PROTEIN *N*-MYRISTOYLTRANSFERASE

98 The post-translational modification (PTM) of proteins contributes hugely to the chemical and
99 functional diversity of the cellular proteome and results in the incorporation of molecular motifs not
100 directly encoded by the genome. Protein *N*-myristoylation is the attachment of a 14-carbon saturated
101 fatty acid, myristate, to the N-terminal glycine residue in a specific set of cellular proteins, catalysed
102 by the enzyme myristoyl CoA:Protein *N*-myristoyltransferase, NMT (Fig. 3) (Wright *et al.* 2010).
103 Whilst *N*-myristoylation is often referred to as a PTM, it most commonly occurs co-translationally.
104 Post-translational myristoylation is less well documented but is known to occur following exposure of
105 an internal glycine after cleavage of proteins by caspases during the apoptotic cascade (Zha *et al.*
106 2000). *N*-Myristoylation can be involved in protein stability, protein-protein interaction interfaces and
107 association of proteins with membranes.

108 NMT appears to be ubiquitous in eukaryotes, including fungi (Towler *et al.* 1987; Lodge *et al.* 1994;
109 Shaw *et al.* 2002), insects (Ntwasa *et al.* 1997), plants (Boisson *et al.* 2003), mammals (including
110 mouse, rat, cow and human) and the parasitic protozoa *P. falciparum* (Gunaratne *et al.* 2000), *L.*
111 *major* (Price *et al.* 2003), *L. donovani* (Brannigan *et al.* 2010) and *Trypanosoma brucei* (Price *et al.*
112 2003). NMT has been shown to be essential for survival in the bloodstream form of *T. brucei* (Price *et*
113 *al.* 2003; Price *et al.* 2010), in insect stages of *L. major* (Price *et al.* 2003) and *L. donovani* (Brannigan
114 *et al.* 2010) and most recently in the rodent malaria parasite *P. berghei* (Pino *et al.* 2012).

115 *NMT structure and mechanism*

116 The enzyme catalytic cycle has been well-studied in yeast, and follows a Bi-Bi mechanism (Towler *et*
117 *al.* 1987; Rudnick *et al.* 1991). Myristoyl-CoA (Myr-CoA) binds to the apo-enzyme, inducing a

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118 conformational change that allows the protein substrate to bind. Myristate is transferred by attack of
119 the N-terminal glycine amine of the peptide on the thioester carbonyl of Myr-CoA (Rudnick *et al.*
120 1991); CoA is released followed by the myristoylated substrate. The first crystal structure of an NMT
121 to be published was *Candida albicans* NMT (*Ca*NMT) (Weston *et al.* 1998). Crystal structures of
122 *Saccharomyces cerevisiae*) (*Sc*NMT) (Bhatnagar *et al.* 1998; Farazi *et al.* 2001b), and more recently
123 *Leishmania donovani* NMT, (*Ld*NMT) (Brannigan *et al.* 2010), *L. major, (Lm*NMT) (Frearson *et al.*
124 2010), and the *P. vivax* enzyme (Goncalves *et al.* 2012b), followed. These structures and others
125 provide insight into the binding sites of Myr-CoA, peptide substrates and inhibitors. Published
126 structures are consistent with a highly conserved Myr-CoA binding mode, with Myr-CoA binding in a
127 bent 'question mark' conformation (Fig. 4b). The thioester carbonyl is placed into an 'oxyanion hole',
128 which activates it for nucleophilic attack, and the fatty acyl chain of Myr-CoA inserts into a deep,
129 hydrophobic pocket. The peptide N-terminal glycine ammonium interacts electrostatically with the
130 buried carboxylate of the C-terminal enzyme residue (Farazi *et al.* 2001b), which is responsible for
131 deprotonation of the ammonium so that the generated nucleophilic amine can attack the Myr-CoA
132 thioester (Farazi *et al.* 2001a; Farazi *et al.* 2001b).

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133 *NMT substrate specificity*

134 NMT appears to be highly specific for transfer of C14 fatty acid, tolerating only slight changes to
135 chain length (reviewed in (Wright *et al.* 2010)). However, peptide substrate specificity is complex and
136 there is no definitive "myristoylation motif", beyond the requirement for an N-terminal glycine. This
137 requirement may be mechanism-based: Gordon and co-workers have suggested that rotation of the
138 peptide N-terminal amine about the peptide backbone aligns it for attack on the thioester, and that
139 such a rotation may be hindered for residues with β-substituents, i.e. any amino acid except for
140 glycine (Farazi *et al.* 2001b). Maurer-Stroh and co-workers used crystal structures and biochemical
141 data to develop a myristoylation predictive tool: the MYR predictor (Maurer-Stroh *et al.* 2002a;
142 Maurer-Stroh *et al.* 2002b), which suggested that as many as 17 residues may be involved in substrate
143 recognition by NMT. A second tool for predicting *N*-myristoylation, the Myristoylator, is based upon
144 a different model for prediction (neural networks, or machine learning) but generates similar error
145 rates compared to the MYR predictor (Bologna *et al.* 2004). Both predictors are necessarily based on
146 the set of proteins annotated or predicted to be *N*-myristoylated using sequence similarity in
147 SwissProt. Unlike the acyl-CoA binding site, the peptide pocket is not well conserved across NMTs
148 from different species (Maurer-Stroh *et al.* 2002b). This, together with the observation that NMT is
149 essential for survival, means that this pocket is a target for selective NMT inhibitors
150 (Georgopapadakou 2002; Price *et al.* 2003).

151 PROTEIN *N*-MYRISTOYLATION

152 Myristoylated proteins have been estimated to make up between 0.5 % and 3 % of eukaryotic cellular
153 proteomes, depending on the species and *in silico* model used (Maurer-Stroh *et al.* 2002a; Martinez *et*
154 *al.* 2008). In the majority of myristoylated proteins studied so far, myristate has a role in transient
155 membrane localisation. The 'two-signal' membrane binding model suggests that strong membrane
156 localisation is only achieved when a second feature of the protein complements myristoylation (Resh
157 1994). This may be a second acyl group near the N-terminus, such as palmitate (which is usually
158 attached to the side-chain of cysteine residues), a polybasic cluster of amino acids that interacts with

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159 membrane phospholipid acidic head groups, or a domain that interacts with another membrane-bound
160 protein (Resh 2006a). Membrane binding in some myristoylated proteins is dynamically regulated via
161 so-called myristoyl-switches, as in the ARF-GTPases, where a change in the ligand bound (from GTP
162 to GDP) causes a conformational change that exposes a hydrophobic pocket that binds myristate,
163 sequestering the fatty acyl chain so that it can no longer interact with the membrane bilayer (Goldberg
164 1998). Myristate-mediated membrane binding is clearly an important mechanism and numerous
165 studies have concluded that it often contributes to the function or regulation of the target protein.

166 *Detecting N-myristoylation in protozoan parasites*

167 Predicting and validating the *N*-myristoylation of potential substrate proteins in general is challenging
168 due to the complex substrate specificity of NMT and difficulties inherent in detecting protein
169 lipidation. Some proteins can be assigned as likely NMT substrates based on homology; an example
170 being the ARF-GTPases, a class of proteins present in all eukaryotes, having common roles and
171 known to be *N*-myristoylated in many organisms (Donaldson and Jackson 2011). For protozoan
172 parasite proteins sharing no sequence identity with generic eukaryotic proteins the main recourse is
173 bioinformatic prediction. However, these tools are necessarily based on known *N*-myristoylated
174 proteins, of which few have been reported in protozoan parasites, and many of these predictions still
175 require experimental proof. Demonstrating *N*-myristoylation of a protein in its native context is non-
176 trivial, and thus non-native approaches predominate. A candidate protein is often over-expressed as a
177 GFP or other tagged construct, or the protein of interest is co-expressed with NMT in *E. coli*, and
178 metabolic radiolabelling with myristate or mass spectrometry is used to demonstrate myristoylation.
179 Lipidation of the protein of interest shown by mass spectrometry, radiolabelling or well characterised
180 chemical probes in the wild type parasite constitute the only methods for direct proof of
181 *N*-myristoylation in the native context, whereas lipidation of an overexpressed construct is good
182 evidence. Other data, based on co-expression of the protein with NMT in *E. coli* or the effects of
183 mutagenesis on membrane localisation are merely suggestive of *N*-myristoylation.

184 *Protein myristoylation in Plasmodium species*

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185 Relatively little is known about which proteins are *N*-myristoylated in *Plasmodium* species. *P.*
186 *falciparum* possesses a single NMT isoform (Gunaratne *et al.* 2000) which is able to transfer
187 myristate from Myr-CoA to a peptide substrate based on *Pf*ARF1 (Gunaratne *et al.* 2000).
188 Experimentally studied substrates have roles in life cycle regulation or progression (calcium
189 dependent protein kinase 1 [CDPK1] and Calpain) (Moskes *et al.* 2004; Russo *et al.* 2009a; Russo *et*
190 *al.* 2009b), host cell invasion (45 kDa glideosome associated protein [GAP45]) (Rees-Channer *et al.*
191 2006), trafficking (ARF1) (Leber *et al.* 2009), Golgi function (GRASP1) (Struck *et al.* 2005) and
192 energy metabolism (Adenylate kinase 2, AK2) (Rahlfs *et al.* 2009). However, only GAP45 and
193 CDPK1 have been shown to be *N*-myristoylated in their native context (Moskes *et al.* 2004; Rees-
194 Channer *et al.* 2006), whilst evidence for *N*-myristoylation of Calpain is based on radiolabelling of a
195 Calpain-GFP construct (Russo *et al.* 2009a). For other potential targets such as Armadillo repeats only
196 protein (ARO), AK2 and GRASP1, evidence is limited (Struck *et al.* 2005; Rahlfs *et al.* 2009;
197 Cabrera *et al.* 2012). CDPK1 requires *N*-myristoylation for membrane localisation (Moskes *et al.*
198 2004), and its gene cannot be knocked out in *P. falciparum* or the rodent parasite *P. berghei*, implying
199 essentiality (Kato *et al.* 2008; Tewari *et al.* 2010). In addition, CDPK1 has key functions in multiple
200 stages of the parasite life cycle and is involved in translational activation during sexual development
201 (Sebastian *et al.* 2012). Another potentially myristoylated kinase, CDPK4, has been shown to be
202 essential for sexual reproduction and mosquito transmission in *P. berghei* (Billker *et al.* 2004).
203 GAP45 is an *N*-myristoylated protein with a direct role in parasite invasion of RBCs. It is localised at
204 the inner membrane complex (IMC), a series of membrane structures lying beneath the parasite
205 plasma membrane (PM) (Jones *et al.* 2006). An actomyosin motor, located between the IMC and the
206 PM, drives merozoite invasion, allowing the parasite to enter the RBC (Baum *et al.* 2006). In
207 *Toxoplasma gondii*, GAP45 is essential to the function of the motor, and therefore for host cell egress,
208 motility and invasion: it has a role in the recruitment of the motor complex and there is evidence for a
209 structural role in maintaining pellicle cohesion during invasion, presumably holding the IMC and PM
210 together (Frenal *et al.* 2010). Recent data on the localisation of *Pf*GAP45 and N- or C-terminal
211 mutants are consistent with this role in spanning the IMC-PM gap (Ridzuan *et al.* 2012).

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212 *Protein myristoylation in Leishmania species*

213 As with *Plasmodium*, very few proteins have been experimentally validated as *N*-myristoylated in
214 *Leishmania* species. Known NMT substrates include proteins involved in trafficking (ARL1 (Sahin *et*
215 *al.* 2008)) and proteins of unknown function, such as HASPB, a member of a family of hydrophilic
216 acylated surface proteins expressed in the host and required for parasite development in the insect
217 vector (Denny *et al.* 2000; Sadlova *et al.* 2010). A family of 'small myristoylated proteins' with
218 probable functions at the flagellum (Tull *et al.* 2004; Tull *et al.* 2012) and a protein phosphatase
219 (PPEF) (Mills *et al.* 2007) have also been reported. In the related trypanosomatid parasites
220 *Trypanosoma brucei* and *T. cruzi*, known likely substrates include *Tb*ARF1 (Price *et al.* 2007),
221 *Tb*ARL1 (Price *et al.* 2005), *Tb*ARL6 (Price *et al.* 2012), cytoskeletal protein *Tb*CAP5.5 (Hertz-
222 Fowler *et al.* 2001), which is involved in cell morphogenesis (Olego-Fernandez *et al.* 2009), a
223 flagellar-localised protein (*Tc*FCaBP) (Godsel and Engman 1999) and a metacaspase implicated in
224 virulence (Proto *et al.* 2011). *N*-Myristoylation of native protein has only been demonstrated directly
225 for *Ld*SMP1 (Tull *et al.* 2004), *Tc*FCaBP (Godsel and Engman 1999), *Tb*CAP5.5 (Hertz-Fowler *et al.*
226 2001) and *Tb*ARL6 (Price *et al.* 2012), but there is evidence for lipidation of *Lm*PPEF (Mills *et al.*
227 2007) and HASPB (Denny *et al.* 2000) in *Leishmania.* In all other cases evidence is limited.
228 Myristoylation of *Ld*ARL1 is essential for localisation to the Golgi (Sahin *et al.* 2008) and ARL3,
229 which is involved in maintaining the flagellum of promastigotes, is also thought to be myristoylated
230 (Cuvillier *et al.* 2000). A number of other proteins have been shown to carry a dual acylation motif,
231 including HASPB, which localises to the outer leaflet of the plasma membrane in infective stages
232 (Denny *et al.* 2000). N-terminal *N*-myristoylation and internal *S*-palmitoylation are both required for
233 this targeting. Similarly, SMP-1 is flagellum-targeted by myristoylation and palmitoylation (Tull *et al.*
234 2004). A bioinformatic approach predicted around 60 *N*-myristoylated proteins in *Leishmania* (Mills
235 *et al.* 2007), many of which are of unknown function and share little identity with other eukaryotic
236 proteins, suggestive of parasite-specific roles.

237 *Current challenges in defining the N-myristoylated parasite proteome*

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238 The handful of identified NMT substrates reflects what is known in other organisms: that *N*-
239 myristoylation is involved in crucial cellular processes. However, a comprehensive understanding of
240 the *N*-myristome and the roles of *N*-myristoylated proteins in protozoa, particularly in infective stages,
241 is still lacking. This is partly due to technical limitations associated with these organisms – for
242 example, challenges in the genetic manipulation of *P. falciparum* and *L. donovani* intracellular human
243 infective stages, and a lack of functional analysis of many of the target proteins – but also because
244 detecting lipidation of a native protein is inherently difficult. Radiolabelling with [^{3}H]myristate or
245 other fatty acids such as palmitate with detection by fluorography is the most common traditional
246 method for studying protein acylation, but is a laborious process due to the very long (weeks or
247 months) exposure times required. Mass spectrometry is a powerful and continually advancing
248 technique that can be used to detect PTMs such as fatty acylation, but generally the protein of interest
249 must first be highly enriched and lipophilic fatty acylated proteins can be lost during preparation
250 (Resh 2006b). The concentration range of proteins within cells is huge – around 5-6 orders of
251 magnitude (Tyers and Mann 2003) – and this further hinders detection of rare proteins in complex
252 mixtures. Chemical proteomic approaches have revolutionised the field of post-translational
253 modification in the past decade, particularly for low abundance PTMs such as *N*-myristoylation. Acyl
254 biotin exchange chemistry (ABE), where palmitate is exchanged site-specifically for biotin at the site
255 of PTM (Roth *et al.* 2006), is widely used for analysing *S*-palmitoylation and was recently applied in
256 *P. falciparum* blood stages with the identification of several thousand new potential targets (Jones *et*
257 *al.* 2012). Another methodology, the bioorthogonal probe approach, involves the metabolic
258 incorporation of a PTM substrate analogue containing a small, biologically inert chemical tag into
259 proteins in live cells; the tag is subsequently functionalised with useful labels for detection or
260 identification (Wright *et al.* 2010; Hang *et al.* 2011). These approaches have been used to great effect
261 for the profiling of acylated proteins in mammalian cells and tagged lipid analogues were recently
262 applied to identify *S*-palmitoylated proteins in *P. falciparum* (in parallel with ABE) (Jones *et al.* 2012)
263 and to demonstrate *N*-myristoylation of *T. brucei* ARL6, a protein with a putative role in flagellum
264 biogenesis (Price *et al.* 2012). Chemical probes have the potential to greatly expand the list of *N*-

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265 myristoylated proteins in parasitic protozoa and contribute to our understanding of NMT as a drug
266 target.

267 TOWARDS NMT INHIBITORS AS ANTIMALARIAL OR ANTILEISHMANIAL AGENTS

268 Several lines of evidence suggest that NMT is a promising drug target for malaria and leishmaniasis;
269 it is a monomeric enzyme carrying out a specific modification on substrates involved in diverse and
270 essential pathways, it is essential for viability where genetic validation has been possible, and is
271 constitutively expressed. Furthermore it is genetically and chemically validated as a drug target in *T.*
272 *brucei*, where small molecule inhibitors have been shown to be effective in animal models (Frearson
273 *et al.* 2010). The wide variety of NMT substrates may limit the potential for resistance to develop
274 against inhibitors targeting the protein binding site, since mutations in this site could inhibit correct
275 myristoylation of substrates.

276 Initial research within our group focused on the discovery of plasmodial NMT inhibitors as chemical
277 probes and potential therapeutic agents for the treatment of malaria, due to the availability of chemical
278 starting points for this indication. Drug repositioning, the adaptation of an existing drug for a new
279 indication, is often used to bypass the significant cost of clinical trials as the safety/pharmacokinetic
280 data for these compounds has already been established (Sleigh and Barton 2010). A similar approach
281 can be used for hit discovery (the “piggy-back approach”) to discover promising hit series without the
282 cost of an HTS campaign (Gelb *et al.* 2003). In the case of NMT, a wealth of information has been
283 collated with the purpose of generating antifungal NMT inhibitors. It was hypothesised that this
284 information could be used as a valuable resource in the generation of parasitic NMT inhibitors, since
285 *Ca*NMT and *Pf*NMT display 38% identity and 65% similarity.

286 In the process of validation of a radioactive assay for monitoring *N*-myristoylation, a library of 43
287 *Ca*NMT inhibitors containing a benzothiazole scaffold (provided by Pfizer) were screened against
288 *Pf*NMT and *Homo sapiens* NMT1 (*Hs*NMT1) (Bowyer *et al.* 2007). Of these 43 compounds, dose-
289 response curves were generated for 7 of the most promising inhibitors, four of which reduced
290 parasitaemia *in vitro*. These compounds had weak enzyme affinity and cellular potency, and displayed

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291 very high molecular weight and lipophilicity for compounds with this level of activity. Ligand
292 Efficiency (LE) is a commonly-used measure of how tightly a compound binds to a target protein,
293 relative to its overall size (Hopkins *et al.* 2004; Bembenek *et al.* 2009); LE > 0.35 is considered
294 favourable, as it suggests scope for substantial optimisation. However, LE in this series was around
295 0.21, limiting the potential for future development. A distinct small library of 25 previously described
296 *Ca*NMT and *Tb*NMT inhibitors was also tested against *Pf*NMT *in vitro* and compound **RO-09-4609**
297 emerged as a promising hit compound, with weak but selective *Pf*NMT affinity. This hit was
298 optimised by iterative medicinal chemistry, resulting in a moderate affinity and highly selective
299 compound **Example 26** (Yu *et al.* 2012), representing a 100-fold affinity improvement over the initial
300 hit (Fig. 5A). The binding mode of these compounds was validated in *Pv*NMT (81% sequence identity
301 to *Pf*NMT), confirming the hypothesis that these compounds are competitive with the peptide
302 substrate; however, LE (0.29) remained sub-optimal. A scaffold hopping approach was then applied
303 to yield 2,3-benzothiophene **Example 9** (Rackham *et al.* 2012), resulting in the most ligand efficient
304 inhibitor discovered at this stage of development against *Pf*NMT (Fig. 5A). Crystallography of this
305 inhibitor series bound to *Pv*NMT confirmed that the 2,3-benzothiophene inhibitors occupy a distinct
306 binding mode to the 2,3,4 benzofurans exemplified by **Example 26** (Yu *et al.* 2012), representing a
307 novel inhibitor series for further development (Fig. 5B) (Rackham *et al.* 2012).

308 Whilst the "piggy-back" approach provided a highly ligand-efficient series of *Pf*NMT inhibitors,
309 cross-screening the same set of compounds against *L. major* NMT failed to identify any starting
310 points (Panethymitaki *et al.* 2006), necessitating alternative hit generation strategies. High-throughput
311 screening has been successful in identifying multiple series of NMT inhibitors, including *C. albicans*
312 (Masubuchi *et al.* 2001; Ohtsuka 2003)*, Aspergillus fumigatus* (Bowyer *et al.* 2008), *Cryptococcus*
313 *neoformans* (Bowyer *et al.* 2008) and *T. brucei* (Frearson *et al.* 2010). Although compounds from the
314 latter publication have been co-crystallised with *L. major* NMT and display excellent enzyme affinity,
315 the cellular potency of this series has not been disclosed. Consequently, we initiated a broader
316 screening programme to identify additional selective inhibitors of *Plasmodium* and *Leishmania*
317 NMTs. We screened the 150,000 compound Pfizer Global Diverse Representative Set against

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318 *Ld*NMT, anticipating that hits would be likely to possess pan-*Leishmania* NMT activity since the
319 residues involved in the binding pocket are completely conserved (Brannigan *et al.* 2010). We also
320 screened the same set against *Pf*NMT, and the initial hit set was supplemented by analogues selected
321 from the remainder of the Pfizer file (~ 2.5 million compounds at the time of the high-throughput
322 screen) (Bell *et al.* 2012). In addition, a separate library of 60,000 compounds was screened against
323 *Pv*NMT, in collaboration with MRC Technology, in the hope of identifying distinct hit compounds
324 (Goncalves *et al.* 2012a).

325 Comparison of the hits from each HTS (Fig. 6) shows a remarkably wide range of structural features.
326 Although many possess a basic centre, a common pharmacophore of several previously described
327 NMT inhibitors, the hits feature primary, secondary and tertiary amines, which may be interacting
328 with the conserved C-terminal Leu of the enzyme. However, the MRCT HTS hit (**MRT00057965**) is
329 not basic and makes an alternative H-bond interaction with Ser319 (Goncalves *et al.* 2012b). Based on
330 the diversity of known binding modes for NMT inhibitors, it is difficult to predict those for novel
331 inhibitor series, and structures of the remaining HTS hits are, as yet, unreported. A limited medicinal
332 chemistry optimization programme based on the MRT hit has been published (Goncalves *et al.*
333 2012b), resulting in a confirmed hit with improved physical properties and moderate selectivity over
334 the human isoforms. In order to address the enzyme selectivity issue, the Pfizer screen hit set was also
335 tested in dose-response assays against both human NMT isoforms and against *T. brucei* NMT (Bell *et*
336 *al.* 2012). Consistent with our previous findings, *Ld*NMT seems distinct from the other NMTs in our
337 screening panel, as most hits show excellent selectivity. In contrast, selectivity for *Pf*NMT over either
338 human NMT is more elusive, though the screen did identify two hit series with encouraging profiles.

339 The structural basis for the observed selectivities remains unclear. Analysis of the peptide-binding
340 pocket of *Ld*NMT identified two residue differences with *T.brucei* NMT (Brannigan *et al.* 2010), and
341 the same residues are also points of differentiation between *Ld*NMT and both human NMTs. In
342 contrast, the residues lining the binding pocket are conserved between *Pf*NMT and human NMT with
343 the exception of a conservative F334Y change, which is also a point of difference between *Pf*NMT
344 and *P.vivax* NMT. *Plasmodium*/human NMT selectivity has been attributed to differences in their

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345 ability to tolerate a conformational change in Y296 (*Hs*NMT1 numbering) (Yu *et al.* 2012). It remains
346 to be seen whether this difference in conformation is observed with other *Plasmodium*-selective
347 inhibitors.

348 FUTURE PERSPECTIVES

349 Two significant hurdles remain for the validation of NMT as a drug target in malaria and
350 leishmaniasis: the challenge of understanding the role of *N*-myristoylation through understanding of
351 its downstream protein substrates, and proving its essentiality in clinically-relevant parasites.

352 Protein *N*-myristoylation is a mechanism used universally by eukaryotes to direct protein localisation
353 and hence function, but detecting protein lipidation by traditional methods presents challenges,
354 particularly in *P. falciparum* and *L. donovani* which are not easily genetically manipulated and go
355 through distinct life cycle stages in a variety of host environments. Bioinformatic prediction suggests
356 that many proteins with diverse and unknown roles are likely to be myristoylated in parasitic
357 protozoa, but in very few cases has acylation been experimentally validated. The development of new
358 techniques to profile protein lipidation, such as the application of bioorthogonal chemical probes
359 (Heal and Tate 2010), may allow us to gain a much wider and in-depth understanding of the
360 myristoylated proteome and to explore the downstream effects of NMT inhibition in these important
361 human pathogens.

362 Demonstration of essentiality and druggability of NMT in *P. falciparum* and *L. donovani* requires
363 selective inhibitors, and proof that these compounds act on-target in parasites. Progress towards
364 selective and potent parasite NMT inhibitors is at an exciting stage; **Example 9** (Fig. 5) derived from
365 the “piggy-back” approach represents a promising starting point for inhibitor development,
366 demonstrating excellent ligand efficiency and selectivity over the human orthologues. Future
367 development of this series will focus on affinity enhancements whilst maintaining optimum
368 physicochemical properties for drug-like molecules, with the aim of generating a high value lead
369 series for clinical development. Replacement of the alkyl ester in **Example 9** with a more biologically
370 stable isostere is a paramount objective for further development since oral administration is a

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371 prerequisite for a malaria medication. In addition, the other *Plasmodium*-selective hits identified (Fig.
372 6) represent highly promising series for further development, demonstrating a range of chemotypes
373 with varying physicochemical properties. Successful elaboration of any of these hit series into a
374 potent, selective and drug-like inhibitor of NMT would enable investigation in cellular and *in vivo*
375 models of malaria, providing a chemical tool for the validation of NMT as a drug target in malaria
376 infections. The need for new drugs against leishmaniasis is still more pressing, particularly in view of
377 the relatively neglected nature of this disease. Our recent screening initiative has also opened the door
378 to the development of potent, selective inhibitors of *Leishmania* NMT, and we anticipate that the
379 available chemical matter will enable both the development of tools to explore the role and
380 importance of NMT in this challenging organism, and new starting points for discovery of
381 antileishmanial drugs.

382 The historic track record of targeted approaches to treatment of parasitic infections is in general
383 relatively poor, since there are significant barriers to overcome in achieving good translation of
384 enzyme to cellular activity and *in vivo* efficacy. Future work in our laboratory is focused on
385 overcoming these hurdles to confirm the relevance of NMT inhibition as a valid target for treating
386 parasitic infections.

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743

744 FIG. LEGENDS

745 **Fig. 1:** Structures of clinically-relevant antimalarial drugs.

746 **Fig. 2:** Structures of clinically-relevant antileishmanial drugs**.**

747 **Fig. 3**: The transfer of myristate from myristoyl-CoA to the N-terminal glycine residue of a target peptide (grey circles) by
748 the enzyme *N*-myristoyltransferase (NMT).

749 **Fig. 4**: A) Ternary co-crystal structure of *Sc*NMT (grey surface) with bound peptide substrate (green) and a non-
750 hydrolysable myristoyl-CoA analogue (cyan) bound in the active site. PDB ID: 1IID, Farazi *et al.*(Farazi *et al.* 2001b). B)
751 Myr-CoA or **NHM** from co-crystal structures with different NMTs; 4A95: NHM in *Pv*NMT, green; 2WUU: NHM in
752 *Ld*NMT, cyan; 2P6E: Myr-CoA in *Sc*NMT, yellow; 1IIC: Myr-CoA in *Sc*NMT, purple; 3IWE: Myr-CoA in *Hs*NMT1,
753 orange; 1IYK: Myr-CoA in *Ca*NMT, blue. Images generated using PyMOL (DeLano Scientific).

754 **Fig. 5:** A) Summary of the *Pf*NMT inhibitor series obtained by a "Piggy-Back" approach from fungal NMT inhibitor RO-
755 09-4609. B) Binding mode of **Example 26 (Yu *et al.* 2012)** bound to the *Plasmodium vivax* NMT active site. PDB
756 Accession Code: 4B14.

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757 **Fig. 6**: Structures of hits obtained by high throughput screening against protozoal NMTs

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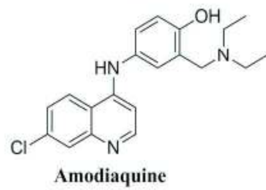
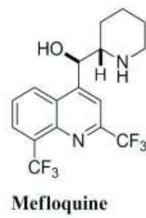
Haem Detoxification Disruptors

Chloroquine


Amodiaquine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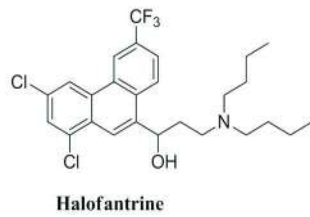
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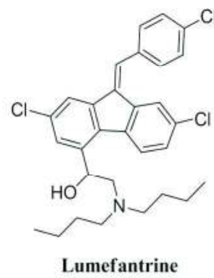
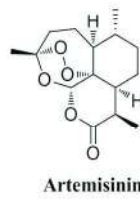
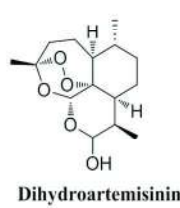
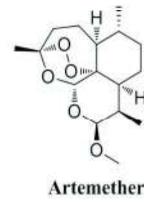
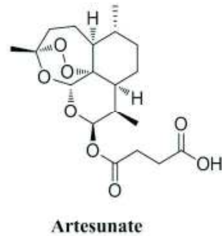

Lumefantrine

Sesquiterpene Lactone Derivatives


Artemisinin


Dihydroartemisinin

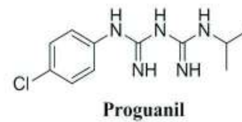

Artemether


Artesunate

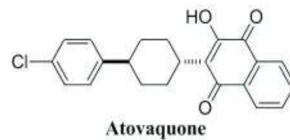
Pyrimidine Biosynthesis Inhibitors

Pyrimethamine

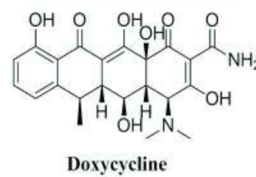
Sulfadoxine


Proguanil

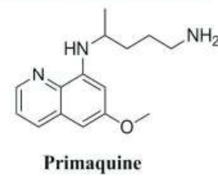
Mitochondrial Electron Transport Disruptor


Atovaquone

Apicoplast Targeting


Doxycycline

Unknown Mechanism


Primaquine

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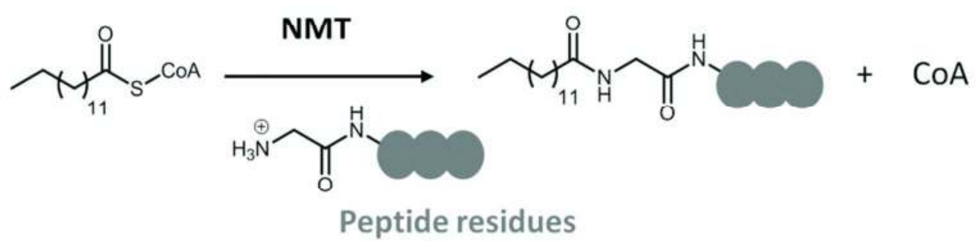
For Peer Review



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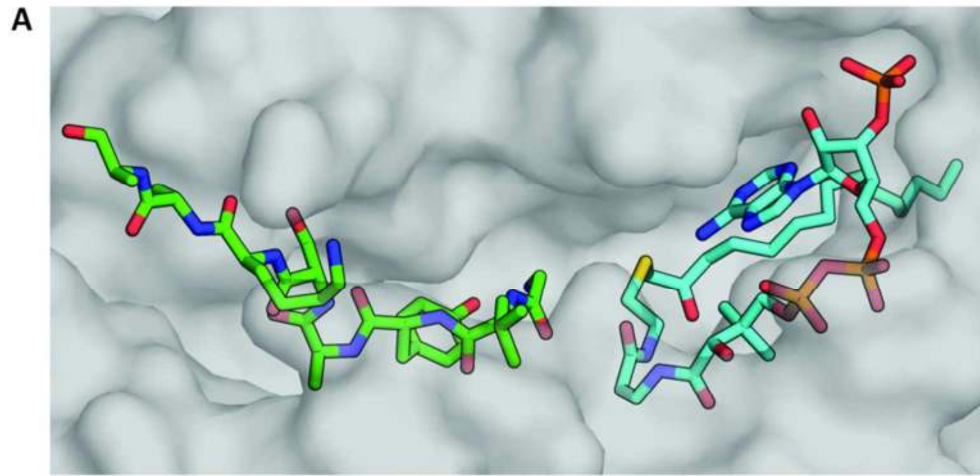
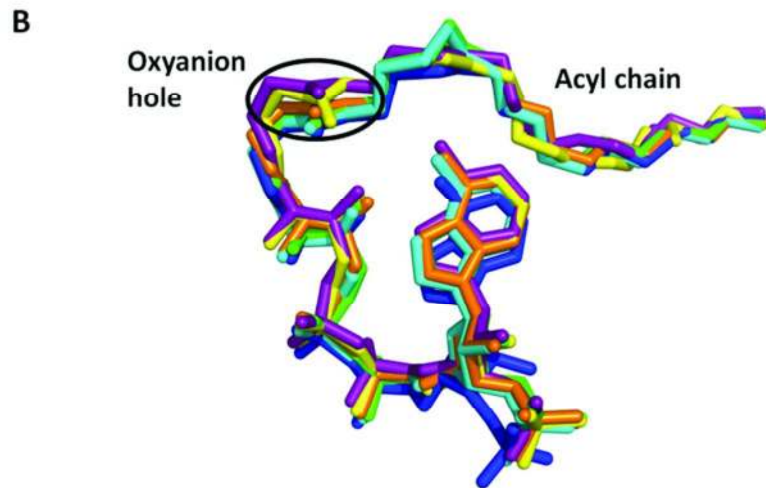
For Peer Review

A



B



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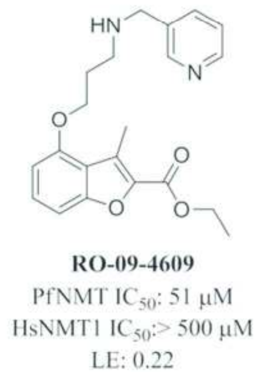
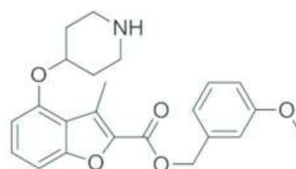
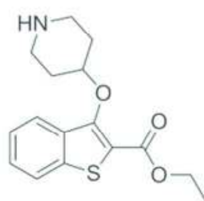
A



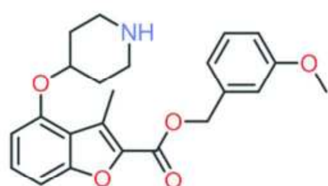


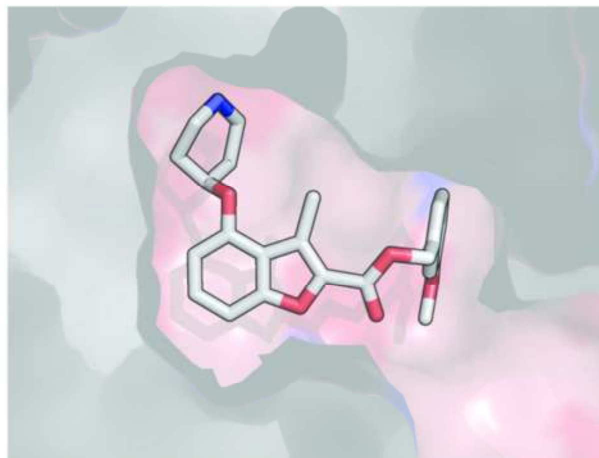
Example 26 (Yu 2012)
PfNMT IC_{50}: 0.60 μM
HsNMT1 IC_{50}:> 100 μM
LE: 0.29



Example 9 (Rackham 2012)
PfNMT IC_{50}: 1.30 μM
HsNMT1 IC_{50}:> 100 μM
LE: 0.38

B





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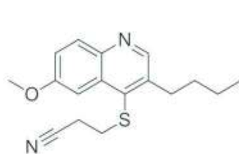
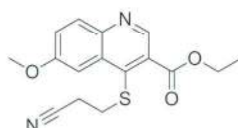
Pv Selective Hits

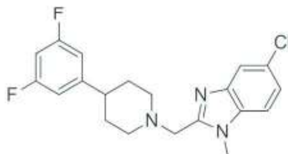


MRT00057965
PvNMT IC_{50}: 2.9 μM

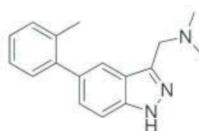


Example 7 (Goncalves 2012b)
PvNMT IC_{50}: 4.7 μM
Hs1NMT IC_{50}: 19.4 μM

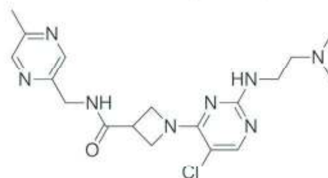
Pf Selective Hits



PF-03665853
PfNMT IC_{50}: 0.91 μM
LdNMT IC_{50}: > 80 μM

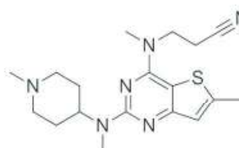


PF-02378143
PfNMT IC_{50}: 2.54 μM
LdNMT IC_{50}: 65.4 μM

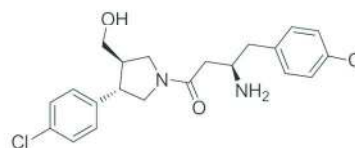


PF-03431814
PfNMT IC_{50}: 0.48 μM
LdNMT IC_{50}: 72 μM

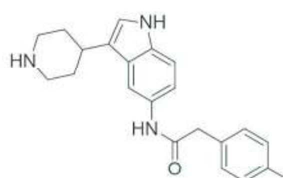
Ld Selective Hits



PF-00349412
PfNMT IC_{50}: 39.6 μM
LdNMT IC_{50}: 0.48 μM



PF-03402623
PfNMT IC_{50}: 71.9 μM
LdNMT IC_{50}: 0.093 μM



PF-03393842
PfNMT IC_{50}: 9.0 μM
LdNMT IC_{50}: 0.10 μM

166x162mm (300 x 300 DPI)