Characterization and selective inhibition of myristoyl-CoA:protein N-myristoyltransferase from *Trypanosoma brucei* and *Leishmania major*

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The eukaryotic enzyme NMT (myristoyl-CoA:protein N-myristoyltransferase) has been characterized in a range of species from *Saccharomyces cerevisiae* to *Homo sapiens*. NMT is essential for viability in a number of human pathogens, including the fungi *Candida albicans* and *Cryptococcus neoformans*, and the parasitic protozoa *Leishmania major* and *Trypanosoma brucei*. We have purified the *Leishmania* and *T. brucei* NMTs as active recombinant proteins and carried out kinetic analyses with their essential fatty acid donor, myristoyl-CoA and specific peptide substrates. A number of inhibitory compounds that target NMT in fungal species have been tested against the parasite enzymes *in vitro* and against live parasites *in vivo*. Two of these compounds inhibit

TbNMT with IC₅₀ values of < 1 µM and are also active against mammalian parasite stages, with ED₅₀ (the effective dose that allows 50 % cell growth) values of 16–66 µM and low toxicity to murine macrophages. These results suggest that targeting NMT could be a valid approach for the development of chemotherapeutic agents against infectious diseases including African sleeping sickness and Nagana.

Key words: drug target, enzyme kinetics, *Leishmania major*, myristoyl-CoA:protein N-myristoyltransferase, N-myristoylation, *Trypanosoma brucei*.

INTRODUCTION

Co-translational modification of proteins by N-myristoylation is a mechanism utilized by eukaryotes to facilitate membrane attachment and protein–protein interactions [1,2]. Catalysed by the enzyme NMT (myristoyl-CoA:protein N-myristoyltransferase), this process involves the covalent attachment of myristic acid to the N-terminal glycine residue of target proteins, which include signalling cascade components and structural proteins [2]. NMT catalysis proceeds via an ordered Bi Bi reaction mechanism [3]. Initial binding of myristoyl-CoA to the enzyme causes a conformational change, leading to the formation of a peptide-binding site and generation of a ternary myristoyl-CoA:NMT:peptide complex. Following catalysis, the N-myristoylated product is released. NMTs have been characterized in a range of eukaryotes, including *Saccharomyces cerevisiae* [4], *Plasmodium falciparum* [5] and human cells [6], and shown to be essential for viability in a number of human pathogens, including the fungi *Candida albicans* and *Cryptococcus neoformans* [7], and the parasitic protozoa *Leishmania major* and *Trypanosoma brucei* [8]. Comparative sequence and biochemical analyses have demonstrated high conservation of myristoyl-CoA-binding sites in human and fungal NMTs but divergent peptide binding specificities [2]. Given these observations, peptide-based and peptidomimetic inhibitors have been developed that show selectivity against the NMTs of pathogenic fungal species as compared with human NMT [9–11]. These results suggest that targeting

NMT could be a valid approach for the development of chemotherapeutics against a range of infectious diseases.

Parasitic kinetoplastid protozoa, including *Trypanosoma* and *Leishmania* species, are major causes of tropical infection worldwide (see <http://www.who.int/tdr/index.html>) and yet only a very limited number of effective drugs are available for use in areas of endemic disease. We have previously used gene targeting and RNAi (RNA interference) to demonstrate that NMT is essential for viability in *L. major* and *T. brucei* [8], suggesting that inhibition of NMT activity in these species might be a useful strategy for a drug development programme [12]. In the present study, we further characterize the NMTs of *L. major* and *T. brucei* and test a panel of compounds developed as fungal NMT inhibitors for their specificity and sensitivity against these parasite enzymes *in vitro* and *in vivo*. Our results suggest that targeting TbNMT (*T. brucei* NMT) may be effective in the development of anti-trypanocidal compounds.

METHODS

PCR amplification, cloning and expression

The TbNMT gene was amplified from genomic DNA using Pfu DNA polymerase (Promega) at 58 °C annealing temperature and the primers, TbNMT_{for} (5'-TTATTATCATATGACTGACA-AAGCATTACG-3') and TbNMT_{rev} (5'-ATTAGGATCCTT-AAACCATCACAAAGAC-3') based on the gene sequence TRYP10.0.001826-6. The NdeI and BamHI sites used to clone

Abbreviations used: ARL1, ADP-ribosylation factor related protein 1; BiP, immunoglobulin heavy-chain binding protein; BSF, bloodstream form; CAP5.5, cytoskeleton-associated protein 5.5; DAPI, 4',6-diamidino-2-phenylindole; DTT, dithiothreitol; ED₅₀, the effective dose that allows 50 % cell growth; GST, glutathione S-transferase; HASPA, hydrophilic acylated surface protein A; IPTG, isopropyl β-D-thiogalactoside; NMT, myristoyl-CoA:protein N-myristoyltransferase; LmNMT, *Leishmania major* NMT; PCF, procyclic form; RNAi, RNA interference; SI, selectivity index; SPA, scintillation proximity assay; TbNMT, *Trypanosoma brucei* NMT; VSG, variant surface glycoprotein.

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the amplified fragment into the vector pET-15b (Novagen) are underlined (as are other restriction sites below). The resulting plasmid, pNMTb, was transformed into *Escherichia coli* BL21(DE3) for expression. The LmNMT (*L. major* NMT) gene was amplified from pNMT [8] using primers LmNMT_{for} (5'-ATACGGATCCTGTCTCGCAATCCATCGAACTC-3') and LmNMT_{rev} (5'-AATACTCGAGCTACAGCATCACCAAGGCAACCT-3') and Pfu DNA polymerase, as above.

The amplified fragment was digested with BamHI and XhoI and cloned into the pGEX-5X-1 vector (Amersham Biosciences). The resulting plasmid, pGNMT, was transformed into *E. coli* BL21(DE3) for expression. The pHASPA expression plasmid has been described previously [8].

The *L. major* HASPA gene was amplified from pHASPA using primers HAWt_{for} (5'-TACACCATGGGAAGCTCTTGCACGAGGAC-3') or HAG2A_{for} (5'-TACACCATGGCAAGCTCTTGCACGAAGGAC-3') and HA_{rev} (5'-AATAAGGATCCCTAGTTCGGGCAGCGT-3'). The amplified fragments were digested with the restriction enzymes NcoI and BamHI and ligated into pET28 vector (Novagen). The resulting plasmids, p28HASPA and p28HASPAm, express wild-type and mutant (G2A mutation) *L. major* HASPA (hydrophilic acylated surface protein A) protein with a C-terminal His tag when transformed into *E. coli* BL21(DE3).

Expression of N-terminally His₆-tagged recombinant TbNMT was induced after growth at 37 °C to A₆₀₀ (absorbance) of 0.6 by addition of IPTG (isopropyl β-D-thiogalactoside) to 1 mM final concentration. After a further 4 h growth at 30 °C, the bacterial cells were lysed in ice-cold buffer A (50 mM Tris/HCl, pH 7.4, and 300 mM NaCl) and centrifuged at 14 000 g for 45 min to pellet insoluble material, prior to affinity chromatography using TALON™ beads (BD Biosciences). The His₆-tagged protein was eluted with imidazole (75 mM imidazole in buffer A) and dialysed extensively to 50 mM Tris/HCl (pH 7.4), prior to Resource™ Q anion-exchange chromatography (Amersham Biosciences). Following gradient elution with 0–1 M NaCl, TbNMT protein was visualized using SDS/PAGE.

Recombinant LmNMT was expressed from pGNMT by addition of IPTG to 1 mM final concentration, following bacterial growth to A₆₀₀ 0.7 at 37 °C. After a further 6 h growth at 18 °C, the bacterial cells were lysed in ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ and 1.7 mM KH₂PO₄, pH 7.4). After centrifugation at 14 000 g for 45 min, the soluble material was added to glutathione–Sepharose 4B beads and incubated at 4 °C for 16 h. The beads were subsequently washed extensively with PBS, followed by a final wash in buffer B (50 mM Tris/HCl, pH 8.0, 1 mM CaCl₂ and 100 mM NaCl). Factor Xa (Amersham Biosciences) was added to the beads (10 cleavage units per mg of fusion protein) and incubated overnight at 4 °C.

After transfer to a PD-10 column (Amersham Biosciences), the flow through was collected and buffer B-exchanged prior to Resource™ Q anion-exchange chromatography and detection by SDS/PAGE. For immunoblotting, proteins were size-separated by SDS/PAGE and transferred on to nitrocellulose membrane (Millipore), prior to probing with anti-His antibody (1:2000; Santa Cruz Biotechnology). Immune complexes were detected using ECL® (Amersham Biosciences).

Functional co-expression of recombinant TbNMT in *E. coli* was carried out as described previously [8].

Parasite culture, immunofluorescence microscopy and *in vivo* inhibition studies

PCF (procyclic form) and BSF (bloodstream form) *T. brucei* parasite strains were maintained as described previously [13]. Im-

munoblotting was used to detect NMT expression in both parasite stages: cells were harvested by centrifugation (800 g for 10 min at 4 °C), washed with ice-cold PBS, resuspended in SDS loading buffer [50 mM Tris/HCl, pH 6.8, 100 mM DTT (dithiothreitol), 2 % (w/v) SDS, 0.1 % Bromophenol Blue and 10 % glycerol], denatured for 5 min by boiling, and total proteins were separated by SDS/PAGE (10 % gel), prior to immunoblotting with the cross-reactive antibody raised against LmNMT [8].

For cell fractionation, washed BSF *T. brucei* cells were lysed in 0.2 M Tris/HCl (pH 8.0), 6 mM MgCl₂, 1 mM EDTA and 1 mM DTT plus complete protease inhibitors (Roche) by mechanical disruption, using 300 mm glass beads (Sigma). Undisrupted cells were cleared by centrifugation at 500 g for 10 min at 4 °C. The supernatant was then subjected to centrifugation at 100 000 g (Beckman TLA 100 rotor) for 1 h at 4 °C for separation of membranes from cytosolic fractions. After precipitation of the proteins with trichloroacetic acid, both fractions were resuspended in SDS loading buffer, separated by SDS/PAGE and immunoblotted.

Indirect immunofluorescence assays were performed essentially as described in [13], using the rabbit primary antibody against LmNMT (1:50 dilution) and Alexa Fluor® 633-conjugated secondary antibody (Invitrogen). Samples were co-stained with DAPI (4',6-diamidino-2-phenylindole) and visualized by confocal microscopy using a Zeiss LSM 510 meta, with a Plan-Apochromat × 63/1.4 Oil DIC I objective lens (where DIC is differential interference contrast). Images were acquired using LSM 510 version 3.2 software (Zeiss).

To test inhibitory compounds, 2 ml of BSF *T. brucei* (at 5 × 10⁴ cells/ml) or *L. major* exponential-phase promastigotes (grown as described in [8]) were grown in 24-well flat-bottom plates in the presence and absence of test compounds, over a period of 72 h. Parasite numbers were counted at regular intervals using a haemocytometer. Alamar Blue® was also used as a colorimetric indicator of viability [14] following parasite incubation with test compounds for 24 h.

The concentration of inhibitor required to reduce parasite growth rate to 50 % as compared with controls [ED₅₀ (the effective dose that allows 50 % cell growth)] was determined. The cytotoxicity of test compounds for murine macrophages (RAW264 cells, maintained at 2 × 10⁵ cells/ml) was also determined using Alamar Blue®.

Potential NMT inhibitory compounds were provided by Pfizer (Sandwich, Kent, U.K.); structural information is provided in Table 2. UK-370485 is a benzothiazole-based compound and has been described previously [15].

In vitro activity assays

LmNMT and TbNMT were assayed using the synthetic octapeptides GQLFTSRR and GCGGSKVK respectively. GQLFTSRR is derived from the N-terminal sequence of *L. major* ARL1 (ADP-ribosylation factor related protein 1) (GQLFTSLN; LmjF04.0340; [13]) but modified at positions 7 and 8 (L7R, N8R) to generate positive charge at pH 7.3. Peptide GCGGSKVK is based on the N-terminal sequence of CAP5.5 (cytoskeleton-associated protein 5.5) [16]. The assay was modified from the method of King and Sharma [17]. In brief, reactions were run in a total volume of 50 µl containing [³H]myristoyl-CoA (Amersham Biosciences) and peptide, and initiated by the addition of TbNMT or LmNMT in 30 mM Tris (pH 7.4), 0.5 mM EDTA, 0.45 mM EGTA, 4.5 mM 2-mercaptoethanol and 1 % Triton X-100. After 10 min incubation at 30 °C, the reaction was terminated by spotting 10 µl on P81 phosphocellulose paper (Whatman). For calculation of the K_m for the ARL1 peptide, 5 ng of purified

LmNMT protein was incubated with 5 μ M [3 H]myristoyl-CoA and 12.8–205 μ M peptide. To determine the K_m for myristoyl-CoA, the peptide concentration was maintained at 103 μ M, while varying the myristoyl-CoA concentration (0.38–9.16 μ M). The procedure was repeated for the TbNMT enzyme using CAP5.5 peptide at 0.32–80.0 μ M. For *in vitro* inhibition assays, the NMT enzymes were incubated with 0.2 mM of compound before reaction initiation by the addition of peptide.

The IC₅₀ values for compounds CP-014553 and CP-030890-27 with TbNMT were determined using the activity assay described above. Different concentrations of each compound were pre-incubated with the enzyme for 10 min at 30 °C before initiating the reaction.

Maximum velocities and K_m values were obtained and fitted to the appropriate kinetic equation using the GraFit package (version 4.04; Erithacus Software; <http://www.erithacus.com/grafit/index.htm>).

SPA (scintillation proximity assay) for NMT activity

An SPA for NMT activity has been developed for inhibitor screening in 96-well plate format. Full details of the assay have been published elsewhere [17a] and are available from P. W. B. on request. Briefly, each 100 μ l reaction contained equal volumes of inhibitor (or buffer alone), NMT, 500 nM [3 H]myristoyl-CoA (8 Ci/mmol; Amersham Biosciences) and 500 nM biotinylated peptide substrate. All solutions were prepared using assay buffer (30 mM Tris, 0.5 mM EGTA, 0.5 mM EDTA, 2.5 mM DTT adjusted to pH 7.4 with HCl and 0.1 % Triton X-100). The reactions were run for 30 min before analysis using streptavidin SPA beads (Amersham Biosciences) and scintillation counting using a Chameleon plate reader (Hidex, Finland).

IC₅₀ values were determined for compounds CP-014553 and CP-005240 over the concentration range 25 μ M–16.4 nM, and data were analysed using the GraFit package. The values quoted are the means derived from triplicate assays.

RESULTS

Expression and localization of TbNMT

The 1341 bp open reading frame of the single copy *TbNMT* gene codes for TbNMT, a 50.5 kDa protein of pI 6.2 that shares > 50 % amino acid identity with NMT from other trypanosomatid species [8], including those amino acids implicated in catalysis, e.g. the glutamic and leucine residues that form the floor of the active site pocket [18]. Because of this high relatedness, antiserum raised against LmNMT also recognizes TbNMT [8]. Hence this reagent could be used to determine the expression pattern of the *T. brucei* enzyme in vector PCFs and mammalian BSFs of the parasite. As might be predicted from RNAi analysis in both parasite stages [8], immunoblotting with anti-LmNMT (Figure 1A) revealed approximately equivalent levels of the 50.5 kDa TbNMT protein in both parasite stages relative to the endoplasmic reticulum marker protein, BiP (immunoglobulin heavy-chain binding protein) (which is expressed at approximately twice the level in BSF as in PCF; [19]). No other proteins were detected by anti-LmNMT in *T. brucei* lysates. Thus TbNMT is constitutively expressed and, most importantly, present in those parasites that cause disease in mammals.

The LmNMT antiserum was also used to determine the cellular localization of TbNMT. Following cell lysis and fractionation, membrane and cytosolic fractions of BSF were analysed by immunoblotting (Figure 1B). Relative to VSG (variant surface glycoprotein), the major GPI (glycosylphosphatidylinositol)-anchored surface component specific to BSFs, NMT is distributed equally

Figure 1 TbNMT is constitutively expressed and partitions between membrane and cytosolic fractions

(A) Total parasite lysates from BSF and PCF *T. brucei* were size-separated and immunoblotted using anti-NMT or anti-BiP antibodies (to monitor protein loading between lanes). (B) Lysed BSF were fractionated into cytosolic (C) and membrane (M) components, prior to size separation and immunoblotting with anti-NMT or anti-VSG antibodies (for detection of the major BSF lipid-anchored surface antigen). (C) Immunofluorescence microscopy of BSF and PCF *T. brucei*. Cells are shown as viewed under phase contrast, visualized for fluorescence with anti-LmNMT (red) and co-stained with DAPI (blue, to reveal positions of the nucleus, n, and kinetoplast, k). Scale bar, 5 μ m.

between membranes and the cytosol, an observation confirmed by immunofluorescence analysis (Figure 1C) and consistent with NMT distribution in *Drosophila*, *Arabidopsis thaliana*, human cells and *Leishmania* [8,20–22], but not in *S. cerevisiae*, in which NMT is predominantly cytosolic [23]. In the micrographs in Figure 1(C), TbNMT fluorescence is excluded from the nucleus in both BSF and PCF parasites but is otherwise distributed throughout the cytoplasm with a punctate appearance, suggestive of partial localization to the endomembrane system and other intracellular sites. Expression levels are also similar in both cell types, in agreement with the immunoblots in Figure 1(A).

TbNMT is active in *E. coli*

A previously described bacterial co-expression assay system [24] was used to demonstrate the activity of TbNMT *in vivo*. Plasmids expressing TbNMT and the LmNMT substrate, HASPA, were

Figure 2 Recombinant TbNMT requires a Gly-2 residue to myristoylate an *L. major* substrate in *E. coli*

(A) pNMTtb and pHASPA were transformed into *E. coli*, either together or separately, and recombinant proteins (50.5 and 17 kDa respectively) were expressed following induction with IPTG (lanes 2, 4 and 6) in the presence of [3 H]myristate. The proteins were size-separated by SDS/PAGE (12% gel) and visualized using Coomassie Blue (upper panel). Three radiolabelled products were detected by autoradiography: the NMT:myristoyl-CoA binary complex (*), the myristoylated HASPA protein (arrowed in A and B) and a myristoylated endogenous *E. coli* protein (lower panel). (B) pNMTtb and p28HASPA/p28HASPA Δ were transformed into *E. coli* as in (A). Three radiolabelled products were detected with p28HASPA (expressing wild-type protein) as in (A) (lane 4). Only two products were detected in the presence of p28HASPA Δ (expressing the *L. major* G2A mutant HASPA protein substrate, Δ), the NMT:myristoyl-CoA binary complex and the endogenous *E. coli* protein (lower panel).

co-transformed into *E. coli*, prior to induction of protein expression from both plasmids and radiolabelling to monitor N-myristoylation [8]. The [3 H]myristate-radiolabelled products were size-separated by SDS/PAGE, followed by Coomassie Blue staining and autoradiography. As shown in Figure 2(A), radiolabelled proteins migrating at 50.5 and 17 kDa were specifically expressed in the co-transformed bacteria (lane 4). These proteins were identified as TbNMT and HASPA respectively by immunoblotting (results not shown). The apo-TbNMT forms a high-affinity complex with myristoyl-CoA [3], explaining the presence of the radiolabelled 50.5 kDa TbNMT. The HASPA was N-myristoylated by the plasmid-encoded TbNMT, yielding a 17 kDa labelled product. Transformation with pHASPA alone (lane 6) gave high protein expression but no detectable radiolabelled product. Thus recombinant TbNMT is active in this heterologous *in vivo* bacterial system and is required for the N-myristoylation of HASPA. Expression of TbNMT in the absence of substrate in this assay resulted in N-myristoylation of an endogenous 25 kDa *E. coli* protein (lane 2), as observed previously [8]. This protein is also N-myristoylated in the presence of the HASPA substrate (lane 4), despite the difference in their expression levels, suggesting that the bacterial protein carries an N-myristoylation motif that is favourable for TbNMT.

The *E. coli* co-expression assay was also used to demonstrate that a glycine residue in position 2 of the HASPA substrate is essential for N-myristoylation to occur. Plasmids expressing wild-type and G2A *L. major* HASPA proteins (p28HASPA/p28HASPA Δ respectively) were co-transformed together with pNMTtb and [3 H]myristate added to the cultures, prior to ana-

lysis of the radiolabelled products as described above. As shown in Figure 2(B), expression of the C-terminally His-tagged proteins was not detectable by staining but the presence of these proteins was verified by immunoblotting, using anti-His antibody (results not shown). Expression of wild-type HASPA in the presence of TbNMT and [3 H]myristate resulted in the expected N-myristoylation of the 17 kDa HASPA (lower panel, lane 2) but expression of the G2A HASPA protein under the same conditions yielded no detectable radiolabelled product (lower panel, lane 4). Radiolabelled TbNMT:myristoyl-CoA binary complex was detected at equivalent levels with both protein substrates. These observations correlate with *in vitro* experimental results demonstrating that replacement of glycine with alanine at the N-terminus removes the ability of peptides to act as substrates for either *S. cerevisiae* or *C. albicans* NMT [25,26].

Purification and kinetic analyses of recombinant *T. brucei* and *L. major* N-myristoyltransferases

The *LmNMT* gene was cloned into pGEX-5X, for expression of LmNMT as a C-terminal fusion with the 26 kDa *Schistosoma japonicum* GST (glutathione S-transferase). The resulting plasmid, pGNMT, was transformed into *E. coli* BL21(DE3) and expression was induced by addition of IPTG. A fusion protein of the expected size (75 kDa) was detected by immunoblotting with anti-NMT antibody (Figure 3A, left panel). This GST-LmNMT protein was purified by affinity chromatography, and NMT was cleaved from the fusion protein using Factor Xa (Figure 3A, left panel, lane 3). The digestion products containing LmNMT were buffer-exchanged prior to anion-exchange chromatography and

Figure 3 Purification of *L. major* and *T. brucei* recombinant NMTs

(A) Recombinant LmNMT was expressed as a GST–NMT fusion protein and purified by affinity chromatography with GST-affinity beads, followed by Factor Xa cleavage and anion-exchange chromatography. Left panel: GST–LmNMT, detected as a 75 kDa protein in SDS/PAGE separations of whole-cell lysates (Coomassie Blue staining, upper lanes 1 and 2) and immunoblotted with anti-NMT antibody (lower lanes 1 and 2). Lane 3, LmNMT (the lower band of 50.5 kDa band) following Factor Xa cleavage. Right panel: further purification of LmNMT by anion-exchange chromatography. Lane 1, starting material; lane 2, wash; lanes 3 and 4, elution fractions, stained by Coomassie Blue (upper panel) or immunoblotted with anti-NMT antibody (lower panel). (B) TbNMT was expressed as a His-tagged fusion protein and purified by affinity chromatography, followed by anion-exchange chromatography as in (A). Left panel: soluble lysate of BL21(DE3) *E. coli* expressing TbNMT (Coomassie Blue staining, upper lanes 1 and 2) was applied to a TALON™ column and bound protein was eluted using an imidazole step gradient (upper lanes 3–6). Immunoblotting with anti-NMT antibody is shown below. Right panel: the imidazole fractions containing TbNMT were pooled and subjected to ion-exchange chromatography with NaCl elution to yield TbNMT purified to apparent homogeneity (E). M, protein markers.

NaCl elution. LmNMT eluted from the column at 100 mM NaCl and was detected as a single band by SDS/PAGE (Figure 3A, right panel).

The *TbNMT* gene was amplified from genomic DNA and cloned into pET15b, for expression as an N-terminally His₆-tagged protein. Following transformation of the plasmid into *E. coli* BL21(DE3), a 50 kDa protein was expressed after IPTG induction and this was recognized by anti-NMT antibody when immunoblotted (Figure 3B, left panel). The TbNMT was initially purified by TALON™ affinity beads (Figure 3B, left panel), prior to buffer exchange and anion-exchange chromatography. As with LmNMT, TbNMT was eluted from the column at 100 mM NaCl and was detected as a single band following SDS/PAGE (Figure 3B, right panel) consistent with a predicted molecular mass of 50.5 kDa.

Kinetic analyses of both recombinant parasite NMTs were carried out using a modified discontinuous assay to monitor the

activities of the purified enzymes [17]. The K_m , V_{max} , k_{cat} and catalytic efficiency (k_{cat}/K_m) values were obtained using octapeptide substrates specific for each enzyme (see the Methods section). Purified TbNMT and LmNMT showed similar affinities for myristoyl-CoA ($K_m = 1.78$ and $1.37 \mu M$ respectively) when calculated using CAP5.5 and ARL1 peptides respectively. These values are in the same order of magnitude as those calculated for human NMT ($9.3 \mu M$) and *S. cerevisiae* NMT ($1.4 \mu M$), when assayed with the substrate GNAAAARR [27]. For TbNMT, the octapeptide substrate demonstrated a K_m of $11.3 \mu M$, while for LmNMT, the ARL1 substrate showed a K_m of $109 \mu M$ (Table 1).

In vitro inhibition of parasite NMT activities using antifungal compounds

Using the indirect discontinuous assay, a range of compounds developed as antifungal agents were tested for their inhibitory

Table 1 Peptide and myristoyl-CoA kinetic parameters for LmNMT and TbNMT

Kinetic analyses of LmNMT and TbNMT were carried out using a modified discontinuous assay [17]. The K_m , V_{max} , k_{cat} and catalytic efficiency (k_{cat}/K_m) values were obtained using the octapeptide substrates, ARL1 and CAP5.5, specific for LmNMT and TbNMT respectively (see the Methods section).

Substrates	LmNMT				TbNMT			
	K_m (μM)	V_{max} ($\mu M/s$)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)	K_m (μM)	V_{max} ($\mu M/s$)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$)
ARL1	110 $\pm$ 33	0.060 $\pm$ 0.006	24.00	0.219	—	—	—	—
CAP5.5	—	—	—	—	11.3 $\pm$ 1.41	0.036 $\pm$ 0.0017	18.2	1.61
Myristoyl-CoA	1.37 $\pm$ 0.29	0.059 $\pm$ 0.01	23.60	17.22	1.78 $\pm$ 0.40	0.076 $\pm$ 0.0059	76.8	43.2

Table 2 *In vitro* inhibition of LmNMT and TbNMT

Compounds originally developed as antifungal agents were tested for their inhibitory activity at 0.2 mM concentration against purified LmNMT and TbNMT (columns 3–5) in the modified discontinuous assay. Two compounds were also tested against TbNMT using the SPA (columns 6 and 7). n.d., not determined.

1 Compound	2 Structure	3 LmNMT activity at 0.2 mM (%)	4 TbNMT activity at 0.2 mM (%)	5 TbNMT IC ₅₀ (nM)	6 TbNMT (SPA) activity at 0.05 mM (%)	7 TbNMT IC ₅₀ (nM)
UK-370485-01		99	104	n.d.	77	n.d.
UK-145974		101	102	n.d.	105	n.d.
CP-030890-27		60	2	250 $\pm$ 40	n.d.	n.d.
CP-014553		58	5	460 $\pm$ 40	7	770 $\pm$ 70
CP-005240		n.d.	n.d.	n.d.	6	740 $\pm$ 80

activity (at 0.2 mM) against purified LmNMT and TbNMT (Table 2). LmNMT activity appeared relatively insensitive to those compounds tested, while more varied effects were observed upon TbNMT activity. For example, as shown in Table 2, UK-370485-01 and UK-145974 did not inhibit TbNMT activity, while CP-030890-27 and CP-014553 were highly effective inhibitors in comparison (with IC₅₀ values of 250 and 460 nM respectively). Notably, the last two compounds were also the only ones that showed any, although weak, inhibition of LmNMT activity (Table 2).

Using the SPA with TbNMT, a similar level of inhibition was obtained for compound CP-014553 (IC₅₀ 770 nM), as well as with an additional compound, CP-005240 (IC₅₀ 740 nM; Table 2).

Antifungal compounds inhibit growth of *T. brucei* BSFs

All anti-fungal compounds were tested for their effect on cell growth and division against cultured *T. brucei* BSF, insect-

stage *L. major* and murine macrophages (RAW267). The ED₅₀ was determined for each compound, as was the SI (selectivity index) for *T. brucei* only (SI is the ratio of ED₅₀ for RAW267 macrophages to ED₅₀ for *T. brucei* BSF), as shown in Table 3. None of the compounds tested was effective against insect-stage *L. major*, including those that showed some inhibitory activity against the LmNMT in Table 2.

However, each of the compounds tested showed activity against *T. brucei* BSF, with the most potent compounds, CP-030890-27 and CP-014553, giving ED₅₀ values of 66 and 16 μM respectively. In addition, CP-030890-27 was remarkably non-toxic to murine macrophages (ED₅₀ > 1500 μM), whereas the SI of CP-014553 was among the highest measured. Growth curves for *T. brucei* incubated with and without these most effective compounds (CP-030890-27 and CP-014553) are shown in Figure 4. Under these conditions, a 50 μM final concentration of compound CP-014553 was lethal within 24 h, whereas CP-030890-27 was tolerated at a higher concentration over the same time period.

Table 3 Selective growth inhibition of BSF *T. brucei* and *L. major* promastigotes

Antifungal compounds described in Table 2 were tested for their effects on growth and division of cultured *L. major* promastigotes, BSF *T. brucei* and murine macrophages (see the Methods section). The ED₅₀ values were determined for each compound. The results shown represent the means $\pm$ S.E.M. obtained from triplicate measurements in two independent experiments. The SI, describing the ratio of ED₅₀ for murine macrophages to ED₅₀ for the parasite, was determined for *T. brucei* only. NE, not effective; M Φ , macrophage.

Compound	ED ₅₀ (μ M)			SI
	<i>L. major</i>	<i>T. brucei</i>	Murine M Φ	
UK-370485-01	NE	160 $\pm$ 21	980 $\pm$ 43	6.1
CP-145974	NE	650 $\pm$ 42	400 $\pm$ 37	0.62
CP-030890-27	NE	66 $\pm$ 6	> 1500	> 23
CP-014553	NE	16 $\pm$ 3	328 $\pm$ 27	21

Figure 4 Effect of anti-NMT compounds on BSF *T. brucei*

Parasites (1×10^5) were incubated in the presence or absence of compounds CP-014553 (**A**) and CP-030890-27 (**B**) over a concentration range and viable parasite numbers counted at the time points indicated, over a 72 h period. Data points were derived from one of three independent experiments and are expressed as $\pm$ S.E.M. for three determinations.

DISCUSSION

Compounds that inhibit NMT activity have been studied intensively as potential antifungal agents, with a principal focus on the *S. cerevisiae* and *C. albicans* enzymes. Both *in vitro* and *in vivo* studies, using myristate and myristoyl-CoA analogues, histidine analogues, aminobenzothiazoles, quinolines and benzofurans to target enzyme activity, have demonstrated the potential for selective inhibition of these and other fungal NMTs [28].

This work has provided the framework for investigation of NMTs from protozoan parasites, with the aim of taking a 'piggyback' medicinal chemistry approach to the development of urgently needed antiparasitic agents [12]. We have previously shown that NMT activity is essential for viability in *Leishmania* and *T. brucei* parasites [8], suggesting that this enzyme may also be an exploitable drug target in these organisms. In the present study, we have expressed and purified recombinant LmNMT and TbNMTs and obtained kinetic parameters for substrates and myristoyl-CoA that correlate with those recorded for fungal NMTs. While the GST tag was removed from LmNMT for this analysis, the TbNMT His tag was not removed. His-tagged proteins have been used routinely in the development of NMT inhibitors [29,30]. Based on known crystal structures, in which His tags are almost always disordered, there is little evidence to suggest that their presence interferes with enzyme activity, a conclusion supported by studies with His-tagged and non-His-tagged *C. albicans* NMT ([30]; P. W. Bowyer, unpublished work).

In vitro studies using four antifungal compounds have demonstrated only weak inhibition of the *L. major* enzyme with these reagents, which also have little effect on parasite growth *in vivo* (results not shown). However, two of the compounds tested (CP-030890-27 and CP-014553) are able both effectively to inhibit TbNMT and also to inhibit the growth of mammalian stages of *T. brucei* in culture. These active compounds are characterized by fused ring systems with particularly oxygen-rich substituents providing opportunities for hydrogen bond formation.

They were identified following high-throughput screening for activity against fungal NMTs and are known to be active against *Ca. albicans* NMT (Pfizer and P. W. Bowyer, unpublished work) but, to our knowledge, are unlike any NMT inhibitory structures so far reported in the literature. These observations provide 'proof-of-concept' for this experimental approach and suggest that the compounds may represent viable leads for future exploitation.

Protein *N*-myristoylation is a two-substrate reaction in which a high-affinity complex is formed between NMT and myristoyl-CoA in the absence of substrate peptide. Upon subsequent peptide binding, reaction occurs followed by the release of free CoA and the myristoylated peptide. In the experiments described here, the K_m values for both parasite NMTs with myristoyl-CoA were in the same range as those previously recorded for both the *S. cerevisiae* enzyme and human NMT1 [27]. The peptides used to determine the K_m values for the parasite enzymes were derived from known *N*-myristoylated protein substrates in these species: for LmNMT, an ARL1-related protein [13], and for TbNMT, a cytoskeletal protein of the calpain family, CAP5.5 [16]. The eight *N*-terminal residues of a peptide or polypeptide substrate appear to be a minimal requirement for binding to the peptide-binding site, and those substrates that have been used to determine K_m values for NMTs vary greatly in their sequence and length [27]. For example, a mutated version of the *N*-terminal peptide of the catalytic subunit cAMP-dependent protein kinase (GNAAAARR) has been used to obtain the K_m for *S. cerevisiae*, *C. albicans* and bovine NMT, yielding 30, 600 and 250 μ M respectively [31]. Using substrate-specific octapeptide substrates for TbNMT and LmNMT, the K_m values reported here, 11.3 and 109 μ M respectively, are comparable. This suggests that the peptide-binding site in these parasitic enzymes shares similar recognition features for binding the *N*-terminal eight residues of peptide substrates as observed for other eukaryotic NMTs [27].

Using the previously described *in vitro* activity assay [17] to test the inhibitory effect of a small number of anti-NMT compounds (Pfizer), we found that none of the compounds tested

was effective in targeting LmNMT enzyme activity. In contrast, two compounds from the same group were able to strongly inhibit TbNMT enzyme activity when used at 0.2 mM. The IC₅₀ values for these reagents were 0.25 µM for CP-030890-27 and 0.46 µM for CP-014553. These compare favourably with IC₅₀ values generated in a screen of non-peptidic and peptidomimetic compounds against *Ca. albicans* NMT [32,33], which were in the range of 0.2–2 µM or less. Initial data using the SPA correlated well with that achieved using the indirect discontinuous assay.

All five of the anti-NMT compounds were also evaluated for their antiparasitic effect against *T. brucei* BSF. Strikingly, the compounds that most effectively inhibited TbNMT *in vitro* were also the most toxic against bloodstream trypanosomes, with ED₅₀ values of 66 and 16 µM (for CP-030890-27 and CP-014553 respectively). These values are comparable with those measured with other putative anti-*T. brucei* compounds such as the 'first generation' farnesyltransferase inhibitors, which have generated ED₅₀ values of 2–50 µM [34]. More recent peptidomimetic farnesyltransferase inhibitors are 1000-fold more effective, with ED₅₀ values of 5–20 nM [35]. Nonetheless, two lead compounds worthy of further investigation have been identified in the present study, on the basis of their inhibitory effect on TbNMT *in vitro*, as well as their inhibitory effect on parasite viability *in vivo*. Both of these show specificity for *T. brucei*, do not inhibit LmNMT and are non-toxic to cultured murine macrophages. These results suggest that inhibition of parasite NMTs may provide another avenue for the development of inhibitory compounds and justify the extension of this work to high-throughput screening and testing against human-infective *T. brucei* species and the intracellular stage of *Leishmania*.

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