Validation of N-myristoyltransferase as Potential Chemotherapeutic Target in Chagas Disease

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VALIDATION OF N-MYRISTOYLTRANSFERASE AS POTENTIAL
CHEMOTHERAPEUTIC TARGET IN CHAGAS DISEASE

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Dedication

To my parents and my future husband who have always supported and encouraged me since the beginning of this journey. And especially to my father who believed in me and pushed me to be my best possible self ever since I was a little girl.

I am here only because of you.
VALIDATION OF N-MYRISTOYLTRANSFERASE AS POTENTIAL CHEMOTHERAPEUTIC TARGET IN CHAGAS DISEASE

by

LINDA JAZMIN HERRERA

DISSERTATION

Presented to the Faculty of the Graduate School of The University of Texas at El Paso in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department of Biological Sciences
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Abstract

The protozoan parasite *Trypanosoma cruzi* is the etiological agent of Chagas disease, a problem endemic to Central and South America. In recent years, this neglected infectious disease has become a global health concern. The only clinically available drugs for the chemotherapy of Chagas disease have important disadvantages such as, high toxicity, strain resistance and variable efficacy. Therefore, there is an urgent need for the development of new antichagasic agents. The enzyme N-myristoyltransferase (NMT) has been characterized in a range of eukaryotes, from *Saccharomyces cerevisae* to *Homo sapiens*. Moreover, NMT has been shown to be essential in protozoan parasites, including *Leishmania major* and *Trypanosoma brucei*. Here, we report the validation of *T. cruzi* NMT as a target and the discovery of lead compounds that specifically inhibit this enzyme. The results from the first part of this dissertation indicate that in *T. cruzi* NMT is constitutively expressed in all stages of the parasite and show at least partial endoplasmic reticulum association. Moreover, we have standardized the heterologous expression and purification of TcNMT for future enzyme kinetics analysis and *in vitro* inhibition studies. In addition, we report for the first time in trypanosomes, an alternative and rapid, non-radioactive method to study protein myristoylation. The second part of this work focuses on the evaluation of NMT inhibitors as anti-*T. cruzi* agents. These compounds were originally designed to target *T. brucei* NMT; here we show their effects on the intracellular parasite *T. cruzi*. Compounds DDD86481, DDD100097 and DDD100144 showed anti-proliferative characteristics in submicromolar concentrations. Moreover, metabolic labeling with myristic acid azide
showed a decreased in the myristoylation of proteins in treated parasite, providing evidence of the “on target” activity of the inhibitors. These inhibitors hold great potential for further exploration as urgently needed new therapeutic agents for Chagas disease.
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Chapter 1: General Introduction

1.1 *Trypanosoma cruzi* and Chagas disease

The flagellate protozoan *Trypanosoma cruzi* is the etiological agent of Chagas disease (or American trypanosomiasis), a problem endemic to Central and South America. Currently, there are 8-11 million infected individuals, resulting in over 15,000 deaths annually. In recent years, Chagas disease has become an emergent concern in the USA due to the immigration from endemic areas, with an estimated 300,000 infected people (Wilkinson and Kelly 2009). Humans and a wide range of other mammals are usually infected when the triatomine vector takes a blood meal defecating at the same time. The metacyclic trypomastigote form of the parasite contained in the fecal material is inoculated through the bite wound or mucous membranes (Bern, Montgomery et al. 2008). The parasite next invades host cells where it transforms into intracellular amastigotes. In this stage they proliferate by binary fission and eventually differentiate into trypomastigotes. The host cell finally ruptures releasing the trypomastigotes into the circulation where they can invade other cells or be ingested in a blood meal by the insect vector. Alternatively, amastigotes that are prematurely released from ruptured cells or derived from extracellular differentiation, can also invade surrounding cells and tissues (McCabe, Remington et al. 1984). In the midgut of the vector the trypomastigote transforms into the replicative form epimastigote. As it travels to the hindgut it differentiates again into metacyclic trypomastigote, and the cycle is completed when the triatomine bug takes a blood meal infecting another mammalian
host (Figure 1.1) (Tanowitz, Kirchhoff et al. 1992). While natural transmission is the most common, it can also be transmitted by blood transfusion and organ transplantation or congenital infection (Epting, Coates et al. 2010). Chagas disease has three stages: acute, indeterminate and chronic. The acute phase is characterized by circulating parasites in the blood and lasts up to 90 days. This phase is generally asymptomatic, although in some cases clinical signs such as, fever, skin lesions, conjunctivitis and enlarged lymph nodes may arise. In a small population, fatal myocarditis or meningoencephalitis can occur, with up to 5% of diagnosed cases resulting in death (Bern, Montgomery et al. 2008; Wilkinson and Kelly 2009). In the indeterminate stage patients are asymptomatic and parasite numbers fall under detectable levels. However, patients are still a reservoir for the parasite, remaining capable of transmitting the disease. Most individuals remain asymptomatic for the rest of their lives; however, about 30% of the patients will progress into the chronic stage of the disease. Manifestations of this stage usually appear 10-20 years after the initial infection, most commonly cardiomyopathy. Less common are the gastrointestinal pathologies of the disease, such as, megacolon and megaesophagus (Bern, Montgomery et al. 2008; Wilkinson and Kelly 2009). The current treatment for *T. cruzi* consists of two nitroheterocyclic derivatives, benzinidazol and nifurtimox (Murta, Gazzinelli et al. 1998). These drugs were developed empirically in the 1960s and 1970s and are very effective in the acute stage of the disease, but have limited activity in the chronic stage of the disease (Croft, Barrett et al. 2005). Moreover, these compounds have severe side effects and the development of invasive lymphomas after treatment with these drugs in rabbits has been reported (Tanowitz, Kirchhoff et al. 1992). In addition, the course of treatment lasts
from 1-4 months, resulting in many cases in incomplete drug schedules, which leads to the development of resistance (Wilkinson and Kelly 2009). These facts clearly point out the urgent need for new drugs to treat Chagas disease.
Figure 1.1. *Trypanosoma cruzi* life cycle.

The bite wound is inoculated with metacyclic trypomastigotes contained in the feces of the triatomine insect (A). Highly motile metacyclic trypomastigotes rapidly invade damaged skin, tissue or mucosa (B). Inside the host cell, the parasite is found in a parasitophorous vacuole (C). Upon parasitophorous vacule rupture, free trypomastigotes undergo a dramatic morphological change into the replicative amastigote form of the parasite (D). The amastigotes continue to divide until they transform back into trypomastigotes (E). Trypomastigotes finally rupture the cell and can be ingested by another triatomine insect in a blood meal or they can infect surrounding cells and tissues (G). Alternatively, extracellular amastigotes released from ruptured cells can also infect neighboring cells or tissues (F). Modified from Lima, Oliveira et al. 2010.
1.2 N-Myristoyltransferase

Myristoyl-CoA:protein N-Myristoyltransferase (NMT) catalyzes the attachment of the fatty acid, myristic acid (C14:0) to the amino-terminal glycine residue of many eukaryotic proteins (Maurer-Stroh, Eisenhaber et al. 2002). The NMT enzyme has an ordered Bi-Bi reaction mechanism where NMT binds first to myristoyl-CoA, which results in a conformational change that allows the peptide to bind next. The myristoyl-CoA:NMT-peptide complex leads to catalysis, and the Co-A product is released first followed by the myristoylated product (Rudnick, McWherter et al. 1991) (Figure 1.2). N-myristoylation was first described as a co-translational modification that occurs in the nascent peptide following the removal of the initiator methionine (Wilcox, Hu et al. 1987). Later, it was established that this modification also occurs post-translationally upon exposure of an internal glycine following caspase cleavage in apoptotic cells (Zha, Weiler et al. 2000). This ubiquitous modification is necessary for the localization and function of several proteins. Moreover, it increases the lipophilicity of proteins; facilitating their association with membranes and promoting protein-protein interactions (Farazi, Waksman et al. 2001). Myristoylation of several proteins involved in cellular regulation and signal transduction; such as, the α subunit of several G proteins and cAMP dependent protein kinases; has been shown crucial for their function (Mumby, Heukeroth et al. 1990; Gaffarogullari, Masterson et al. 2011) It has been validated as an essential protein in Saccharomyces cerevisiae (Duronio, Towler et al. 1989), Candida albicans and Cryptococcus neoformans (Lodge, Johnson et al. 1994). Consequently, NMT has been explored as an antifungal chemotherapeutic target (Masubuchi, Ebiike et
al. 2003). Moreover, previous studies have identified NMT as an appropriate chemotherapeutic target against protozoan parasites including *Leishmania donovani*, *Leishmania major*, *Trypanosoma brucei* and *Plasmodium falciparum* (Bowyer, Gunaratne et al. 2007; Brannigan, Smith et al. 2010; Price, Guther et al. 2010). In *T. cruzi* few N-myristoylated proteins have been experimentally validated, such as, the phosphoinositide-specific phospholipase C and the flagellar calcium-binding protein (Wingard, Ladner et al. 2008; de Paulo Martins, Okura et al. 2010). Nevertheless, bioinformatics analyses have predicted more than 100 proteins to be N-myristoylated (Mills, Price et al. 2007) (Table 1.2.1). In addition, prior myristoylation is necessary before palmitoylation of proteins, another important post-translational modification. Palmitoylation, which is the linkage of palmitic acid (C16:0) to cysteine residues, has been shown to be necessary for sorting to the flagellar membrane in kinetoplastids (Emmer, Souther et al. 2009; de Paulo Martins, Okura et al. 2010). Taking all these facts into consideration, it is expected that NMT inhibition would have pleiotropic effects in the physiology of the parasite. Although this enzyme has been extensively characterized in other kinetoplastids (Price, Menon et al. 2003; Panethymitaki, Bowyer et al. 2006; Brannigan, Smith et al. 2010; Price, Guther et al. 2010), very little is known about *T. cruzi* NMT.
Figure 1.2. *N*-myristoylation reaction mechanism.

Myristoyl-CoA binds first to the enzyme to form the NMT:myristoyl-CoA complex, inducing a conformational change that allows binding of the peptide. Nucleophilic substitution proceeds via attack by the N-terminal glycine amine in the peptide on the myristoyl-CoA thioester. CoA is released first, followed by the myristoylpeptide. Modified from Wright, Heal et al. 2010.
Table 1.1. Classification of kinetoplastid N-myristoylated proteins from high confidence groups. Modified from Mills, Price et al. 2007.

<table>
<thead>
<tr>
<th>Function</th>
<th>No. in L. major</th>
<th>No. in T. brucei</th>
<th>No. in T. cruzi</th>
</tr>
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<tr>
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<td>28</td>
<td>44</td>
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<tr>
<td>Hypothetical proteins (unknown function)</td>
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<tr>
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<tr>
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<td>3</td>
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<tr>
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<tr>
<td>HASPB</td>
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<tr>
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<td><strong>62</strong></td>
<td><strong>123</strong></td>
</tr>
</tbody>
</table>
1.3 Significance and Aims

Historically, *Trypanosoma cruzi* transmission was concentrated in rural areas in Latin America, where the poor housing conditions allowed constant contact with the triatomine insect vector. However, in the last decades large-scale migration from rural areas has brought infected individuals to large cities both within and outside Latin America. In the US, several species of the triatomine bug have been found in the southwest of the country. Moreover, there are as many as 300,000 people living in the US with Chagas disease (Bern, Kjos et al. 2011). The mild and non-specific symptoms that most patients experience, often results in misdiagnosis.

Alarmingly, only two drugs with proven efficacy are available to treat Chagas disease. Nonetheless, neither drug is approved by the US Federal Drug and Food Administration (FDA) and can only be obtained through the CDC and used under investigational protocols. Both drugs are highly toxic and have limited efficacy against established chronic-stage disease, which highlights the urgent need for new chemotherapeutics against *T. cruzi*. This requires the identification of enzymes and metabolic pathways in the parasite that could be used as potential targets for drug development. These enzymes have to be present in the mammalian stages of the parasite. Moreover, given that in the mammal, *T. cruzi* resides mainly intracellularly, the drugs must be able to enter the host cell to reach the parasite. Validation of drug targets and compounds that satisfy these requirements can lead to improved therapies against Chagas disease.
The aims of this dissertation are twofold: 1) To investigate the expression and localization of N-myristoyltransferase in *T. cruzi*, and 2) to evaluate N-myristoyltransferase inhibitors as anti-*T. cruzi* agents.

Previous studies have extensively characterized NMTs from a wide range of eukaryotes. It has been shown to be an essential enzyme in the kinetoplastids *T. brucei*, *L. major* and *L. donovani*. Moreover, selective inhibition of NMT in these parasites leads to parasite death. In mice infected with *T. brucei*, NMT inhibitors cures acute trypanosomiasis. Despite the impressive advances in understanding this protein modification, very little is known about *T. cruzi* NMT. Therefore, the focus of Chapter 2 is to determine if NMT is expressed in all the stages of the parasite, particularly in the mammalian stages, and therefore be considered as a potential chemotherapeutic target. Consequently, Chapter 3 focuses on the chemical evaluation of this enzyme as a target using specific NMT inhibitors. Also, the effects of these inhibitors on *T. cruzi* intracellular proliferation and their “on target” effect are evaluated.

Taken together, the studies described here provide a greater understanding of *T. cruzi* NMT and validate this enzyme as a drug target for Chagas disease. Moreover, they provide a promising start in the characterization of specific inhibitors that will contribute in the critical search for more effective and less toxic drugs against this parasite.
Chapter 2: Expression and Localization of N-myristoyltransferase in

*Trypanosoma cruzi*

### 2.1 Introduction

Myristoylation of proteins is catalyzed by the ubiquitously distributed eukaryotic enzyme *N*-myristoyltransferase. The importance of this modification in the function of proteins involved in signaling or other fundamental cellular processes has brought attention to this enzyme. NMT has been characterized in a range of eukaryotes, including *Saccharomyces cerevisiae* (Duronio, Towler et al. 1989), *Plasmodium falciparum* (Gunaratne, Sajid et al. 2000), *T. brucei* (Panethymitaki, Bowyer et al. 2006), *L. major* (Price, Menon et al. 2003) and human cells (McIlhinney and McGlone 1996). In *Saccharomyces cerevisiae* NMT has been shown to be a cytosolic protein that is not associated with cellular membranes (Knoll, Levy et al. 1992). Conversely, NMTs from *Drosophila* (Ntwasa, Egerton et al. 1997), *Arabidopsis thaliana* (Qi, Rajala et al. 2000) and mammalian cells are known to be partially membrane-associated (Raju, Magnuson et al. 1995; McIlhinney and McGlone 1996). In *L. major* >70% of this enzyme is associated with membranes (Price, Menon et al. 2003), while in *T. brucei* it is equally distributed between membranes and the cytosol (Panethymitaki, Bowyer et al. 2006).

Here we provide the first evidence that *T. cruzi* NMT (TcNMT) is expressed in all the stages of the parasite. Additionally, we demonstrate that NMT is at least partially associated with the endoplasmic reticulum (ER) by colocalization with BiP (binding protein), an ER resident. Moreover, we show that many of the residues predicted to be
implicated in the enzyme mechanism or to have regulatory roles are conserved in TcNMT. We also provide the first insight on the distribution of myristoylated proteins in *T. cruzi* using a bioorthogonal labeling method. Furthermore, we have standardized the heterologous expression and purification of recombinant TcNMT, which will allow for future biochemical characterization of this enzyme and for *in vitro* inhibition studies using larger libraries of compounds.
2.2 Methods

Parasite and mammalian cell culture

LLC-MK2 (green monkey kidney epithelial cells) and U2OS (human osteocytes) (ATCC) cells were culture in high glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated-fetal bovine serum (HI-FBS) at 37°C, under 5% CO₂ atmosphere. Tissue culture cell-derived trypomastigote forms of T. cruzi (TCT) (Y strain) were obtained 5 to 9 days after infection of LLC-MK2 monolayers as previously described (Andrews and Colli 1982). T. cruzi epimastigotes (Epis) (Y strain) were maintained axenically in liver infusion tryptose (LIT) medium at 28°C, as previously described (Camargo 1964).

Purification of intracellular amastigotes (ICA)

LLC-MK2 cells were infected with 10⁸ TCTs. After 5 days, the infected monolayers were gently detached by scraping and resuspended in 5 mLs of phosphate buffered saline (PBS). The suspension containing the infected cells was next transferred to an M-tube (Miltenyi, Biotec). This tube bears a special stator and rotor that allows for tissue homogenization. Also, it has a pre-inserted mesh that retains larger particles, removing them from the homogenized sample. The tube was then placed in the gentleMACS™ Dissociator (Miltenyi, Biotec) for the automated disruption of host cells. The homogenized sample containing the parasites was then passed through a column containing anion exchange preswollen microgranular diethylaminoethyl cellulose (DE52, Whatman). The sialylated TCT forms of the parasites were retained in the resin through
ionic interaction, while the ICA forms were recovered (Marques 2011).

**Immunofluorescence Assay**

Parasites were washed with phosphate buffered saline (PBS) and $10^4$ cells per well were deposited on a 96-well plate, followed by centrifugation at 3500 rpm for 10 min at room temperature. Supernatant was removed and cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature, then permeabilized with 0.1% Triton X-100 in PBS for 10 min. Wells were blocked for 1 hour at room temperature with 3% bovine serum albumin (BSA) in PBS. To obtain polyclonal anti-TcNMT, rabbit sera was rose against the TcNMT peptide RGDGNHLYYFYNSYP (Biomatik). Anti-TcNMT was labeled with Alexa® 594, while rabbit anti-TbBiP (gift from James Bangs, University at Buffalo) was labeled with Alexa® 488, using the APEX Antibody Labeling Kits (Life Technologies) according to the manufacturer instructions. Parasites were incubated overnight with the primary antibodies, both at 1:250 dilution in 3% BSA-PBS. To remove unbound antibody, cells were washed 3X with PBS with 0.1% Tween (0.1% PBS-T). Parasite DNA was then labeled with 4',6-diamidino-2-phenylindole (DAPI) at 0.5 µg/mL. Samples were visualized using a LSM 700 Confocal Microscope (Zeiss) with a 63X oil objective lens. Images were acquired using the ZEN 2009 software (Zeiss).

**Infection of U2OS cells**

Human osteocytes (U2OS cells) were diluted in 10% HI-FBS, DMEM to give a cell density of $2 \times 10^5$ cells/mL. TCTs were resuspended in 10% HI-FBS, DMEM to a final density of $1.2 \times 10^7$ cells/mL. Cells and parasites were mixed at a ratio of 4 volumes of
cells plus 1 volume of parasites. For non-infected cells, U2OS cells were diluted in DMEM, 10% HI-FBS to give a cell density of $2 \times 10^5$ cells/mL. 150 µL of the mixture were dispensed per well. Plates were incubated for 72 or 96 hours at 37°C under 5% CO$_2$ atmosphere. Immunofluorescence assay was performed as described above using anti-TcNMT at 1:250 dilution, followed by anti-rabbit IgG Alexa® 594 conjugated. Host cell and parasite DNA was labeled using DAPI at 0.5 µg/mL. Samples were visualized and images acquired as described above.

**Metabolic labeling and fluorescence microscopy**

$10^7$ parasites were washed with PBS three times and incubated at 37°C in the case of ICA and TCT, and at 28°C in the case of Epis, in 1mL of 2% delipidated bovine serum albumin (BSA), DMEM. After 30 min, 100 µM of myristic acid azide (Life Technologies) was added from 50 mM stock solutions in dimethyl sulfoxide (DMSO). The same volume of DMSO was used as a negative control. Parasites were further incubated for 6 hours. For confocal microscopy analysis, samples were processed as described above. After permeabilization, cells were washed three times with 3% BSA in PBS. “Click” reaction between the myristic acid azide and Alexa Fluor 488® alkyne (Invitrogen) was performed according to the manufacturer instructions using the Click-iT® Cell Reaction Buffer Kit (Life Technologies). Parasite DNA was labeled with DAPI at 0.5 µg/mL. Samples were visualized and images acquired as described above.
**PCR amplification, cloning and expression**

The *TcNMT* gene was amplified from *T. cruzi* Y strain genomic DNA using the primers NMT<sub>fw</sub> (5’-GGATCCATGGCAGAAGGGTTCAGGT-3’) and NMT<sub>rev</sub> (5’-GAATTCTCTAGCATGAACAATCCCACGTC-3’) based on the gene sequence AI069625. The *Bam*HI and *Eco*RI sites used to clone the amplified fragment into the vector pRSETA (Life Technologies) are underlined. The resulting plasmid pNMTtc was transformed in *Escherichia coli* BL21 (DE3) for expression. Expression of N-terminally His<sub>6</sub>-tagged recombinant protein was induced after growth at 37°C to *A<sub>600</sub>* (absorbance) of 0.6 by addition of IPTG (isopropyl β-D-thiogalactoside) to 1 mM final concentration. Expression was confirmed by Coomassie blue staining after SDS-PAGE several time points after induction. After another 12 hours of growth at 18°C, the bacterial cells were lysed in buffer A (20 mM Tris, 1 M NaCl, 10 mM Immidazole) and centrifuged at 18000 g for 45 min to pellet insoluble material, prior to immobilized metal ion affinity chromatography using a HisTrap™ HP column (GE Healthcare Life Sciences). The His<sub>6</sub>-tagged protein was eluted using fast protein liquid chromatography (FPLC) with a gradual increase of immidazole from 10 to 500 mM using buffer B (20 mM Tris, 1 M NaCl, 500 mM Immidazole). *TcNMT* was visualized by Coomassie blue staining after SDS-PAGE and by western blotting using mouse anti-Xpress™ (Life Technologies) at 1:5000 dilution, followed by anti-mouse IgG HRP-conjugated for detection.
2.3 Results

NMT Expression and Localization in *T. cruzi*

To investigate the expression and cellular localization of TcNMT during the *T. cruzi* life cycle, epimastigotes (Epi), intracellular amastigotes (ICA) and tissue cell culture-derived trypomastigotes (TCT) were analyzed by immunofluorescence. Anti-TcNMT was obtained from rabbit sera rose against the TcNMT peptide RGDGNLHYYFYNSYP (Biomatik). Anti-TcNMT showed that NMT is excluded from the nucleus in all three stages and a punctate appearance indicates that it is mostly associated to the endomembrane system and other intracellular sites in the parasite. To further determine the subcellular localization of TcNMT we use anti-TbBiP as an endoplasmic reticulum marker (Bangs, Uyetake et al. 1993). Colocalization with BiP suggests that NMT is at least partially associated to the endoplasmic reticulum (ER) of *T. cruzi* (Figure 2.1). Together, these results demonstrate that TcNMT is constitutively expressed in all the stages of the *T. cruzi* life cycle and that it is associated to the ER. To determine the specificity of anti-TcNMT human osteocytes were infected and analyzed by immunofluorescence. Anti-TcNMT readily labeled intracellular parasites while non-infected cells were not labeled, indicating that there is no cross-reactivity between this antibody and human cells (Figure 2.2). TcNMT shares 59% and 57% overall amino acid identity with NMTs from *T. brucei* and *Leishmania major*, respectively. Therefore, antiserum rose against LmNMT and TbNMT also recognized TcNMT in epimastigotes (Figure 2.3) The expression and distribution of TcNMT is consistent with that seen in *Leishmania* and *T. brucei* (Price, Menon et al. 2003;
Panethymitaki, Bowyer et al. 2006), where NMT is also expressed in both the vector and the mammalian forms of the parasites. Sequence analysis showed that similar to TbNMT and LmNMT, TcNMT is divergent in its N-terminus from all NMTs so far characterized. Interestingly, even though our data suggests ER association, TcNMT does not contain the lysine-rich regions that have been associated with ribosomal targeting in human and mouse NMTs (Glover, Hartman et al. 1997). In contrast, NMT residues that have been predicted to be essential for the activity of this enzyme, including the two negatively charged residues that have been predicted to form the floor of the active site pocket in C. albicans NMT (Johnson, Bhatnagar et al. 1994; Weston, Camble et al. 1998), are conserved in TcNMT. An insertion of ~ 20 amino acids is found close the first “pocket floor” in TbNMT and LmNMT relative to human and fungal NMTs (Price, Menon et al. 2003). In the same localization a 34 amino acid-insertion is observed in TcNMT. An additional insertion in TbNMT and TcNMT is found further downstream. These differences between parasite NMTs and fungal and mammalian NMTs suggest that the enzyme activity might be affected by additional secondary or tertiary structures formed in these parasites NMTs relative to human and fungal enzymes (Figure 2.4).
**Figure 2.1.** TcNMT is constitutively expressed in all *T. cruzi* stages and it is associated to the endoplasmic reticulum.

Immunofluorescence microscopy of Epis, ICAs and TCTs. Cells are shown as viewed under phase contrast, visualized for fluorescence with anti-TcNMT (red) and anti-BiP (green), co-stained with DAPI to reveal positions of the nucleus and kinetoplast (blue). Scale bar, 2 µm.
Figure 2.2. Anti-LmNMT and anti-TbNMT recognize TcNMT.

Immunofluorescence microscopy of Epis stained with anti-LmNMT and anti-TbNMT (red), co-stained with DAPI (blue) to reveal positions of the nucleus and kinetoplast (blue). Scale bar, 5 µm.
**Figure 2.3.** Anti-TcNMT shows no cross-reactivity with human cells.

Immunofluorescence microscopy of non-infected and infected cells 72 and 96 hours post-infection stained with anti-TcNMT (red), co-stained with DAPI (blue) to visualize host cell and parasite DNA. Scale bar, 10 µm.
Figure 2.4. Alignment of *T. cruzi* NMT with NMTs from *T. brucei*, *L. major* and human.

The deduced open reading frame of TcNMT (AI069625) was aligned with TbNMT (TRYP10.0.001826-6), LmNMT (AF3059561) and human NMT (*HUMAN*, P30419) using the Clustaw multiple sequence alignment program (www.ebi.ac.uk/clustaw). Strictly conserved residues are shown in red. The insertions in the parasites NMTs are underlined. Red boxes indicate key residues involved in myristoyl-CoA binding; black boxes indicate residues involved in peptide binding identified in yeast species. Arrows identify the pocket floor residues in *C. albicans.*
Myristic acid azide-labeled proteins are associated to the endomembrane system

It has been shown that metabolic labeling with azido-fatty acid analogs followed by “click” reaction with an alkyne-containing fluorophore, results in fluorescently labeled fatty-acylated proteins (Charron, Zhang et al. 2009). To visualize the distribution of N-myristoylated proteins we first labeled Epis, ICA and TCT with myristic acid azide (Life Technologies). This azide tag is small enough to permit the incorporation of the tagged myristic acid molecule onto proteins by N-myristoyltransferase. In addition, previous studies have shown that treatment with Triton X-100 solubilizes and extracts fatty acid chemical reporters that are not installed onto proteins (Charron, Zhang et al. 2009). Therefore after fixing, we permeabilized with Triton X-100 and performed chemoselective ligation or “click” reaction between the azide and Alexa Fluor® 488 alkyne (Life Technologies) (Figure 2.5). By following this protocol we could ensure that the majority of the Alexa Fluor® 488 signal corresponds mostly to putative N-myristoylated proteins. The IFA showed a punctate appearance indicating endomembrane association (Figure 2.6). A faded signal was detected in the control samples where no myristic acid azide was added. The signal was mostly localized to the nucleus and it probably represents some non-specific Alexa Fluor® 488 alkyne accumulation. Overall, these results are consistent with the notion that N-myristoylation targets proteins to membranes (McIlhinney 1998).
Figure 2.5. Click Azide/Alkyne Reaction.

In the first step myristic acid azide (A) is actively incorporated into the cells. The second step or detection step (B) utilizes chemoselective ligation or “click” chemistry where the azide in the myristoylated proteins reacts with an alkyne-containing dye or hapten. Modified from MOLECULAR PROBES®.
Figure 2.6. Myristoylated proteins are associated to the endomembrane system.
Fluorescence microscopy of Epis, ICAs and TCTs metabolically labeled with DMSO or myristic acid azide were reacted with Alexa® 488 alkyne (green) after permeabilization, co-stained with DAPI (blue) to label the nucleus and kinetoplast. Scale bar, 5 µm.
Expression and purification of recombinant TcNMT

Establishment of robust expression and purification methods for recombinant NMTs from *L. major*, *T. brucei* (Panethymitaki, Bowyer et al. 2006) and *P. falciparum* (Bowyer, Gunaratne et al. 2007) in *E. coli* has allowed for well established fungal NMTs inhibitors to be tested *in vitro*, using a “piggyback” approach (Gelb, Van Voorhis et al. 2003). For future biochemical characterization and *in vitro* inhibition studies, we standardized the expression and purification of the recombinant *T. cruzi* NMT. The *TcNMT* gene was cloned into pRSETA (Life Technologies) for expression of His\(_6\)-tagged enzyme. TcNMT was found in the insoluble fraction of *E. coli* when expressed at 37°C and 30°C. To obtain soluble enzyme, TcNMT was expressed at 18°C, samples taken up to 4 hours post induction were analyzed by SDS-PAGE follow by Coomassie blue staining. Figure 2.7 shows that NMT is mostly present in the soluble fraction. TcNMT was purified by immobilized metal ion affinity chromatography using a HisTrap™ HP column (GE Healthcare Life Sciences) (Figure 2.8). The activity of this recombinant protein remains to be elucidated. However, the standardization of this technique offers a starting point in the biochemical characterization of TcNMT.
Figure 2.7. Heterologous expression of TcNMT in *E. coli*.

Insoluble (A) and soluble (B) fractions of samples obtained at different time points post-induction were analyzed by SDS-PAGE and Coomassie staining.
Figure 2.8. Purification of recombinant TcNMT.

Different elution fractions of TcNMT during purification were visualized by SDS-PAGE followed by Coomassie staining (A) and by western blotting (B).
Chapter 3: Evaluation of N-myristoyltransferase Inhibitors as Anti-\textit{T. cruzi} Agents

3.1 Introduction

The extensive cellular functions of \textit{N}-myristoyltransferase have led to numerous investigations of this enzyme as a target for the development of anticancer, antifungal and antiviral compounds (Georgopapadakou 2002; Hill and Skowronski 2005; Selvakumar, Lakshmikuttyamma et al. 2007). In protozoan parasites a “piggyback” approach has been used, where compounds designed to target cancer and fungal NMTs are tested for their inhibitory properties against parasites (Panethymitaki, Bowyer et al. 2006; Bowyer, Gunaratne et al. 2007).

More recently, potent inhibitors against \textit{T. brucei} NMT (TbNMT) were identified through an initial screening of 62,000 diversity-based compound library. Optimization of the screening hit led to TbNMT inhibitors that prevented the proliferation of bloodstream form of \textit{T. brucei} with a window of selectivity of over 200-fold with respect to mammalian cells (Frearson, Brand et al. 2010; Brand, Cleghorn et al. 2012). Although these compounds presented high inhibition both, \textit{in vitro} and \textit{in vivo}, against the extracellular bloodstream form of \textit{T. brucei}, their effects against intracellular parasites such as \textit{T. cruzi} have not been elucidated. In this study we evaluated the effect of eight of these inhibitors against \textit{Trypanosoma cruzi}. Our results not only demonstrate the potential of the tested compounds as anti-\textit{T. cruzi} agents, but also validate TcNMT as a potential chemotherapeutic target in Chagas disease.
3.2 Methods

Parasite and mammalian cell culture

LLC-MK2 (green monkey kidney epithelial cells) and U2OS (human osteocytes) (ATCC) cells were culture in high glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated-fetal bovine serum (HI-FBS) at 37°C, under 5% CO₂ atmosphere. Tissue culture cell-derived trypomastigote forms of T. cruzi (TCT) (Y strain) were obtained 5 to 9 days after infection of LLC-MK2 monolayers as previously described (Andrews and Colli 1982). T. cruzi epimastigotes (Epis) (Y strain) were maintained axenically in liver infusion tryptose (LIT) medium at 28°C, as previously described (Camargo 1964).

Purification of intracellular amastigotes (ICA)

LLC-MK2 cells were infected with \(10^8\) TCT. After 5 days, the infected monolayers were gently detached by scraping and resuspended in 5 mLs of phosphate buffered saline (PBS). The suspension containing the infected cells was next transferred to an M-tube (Miltenyi, Biotec). This tube bears a special stator and rotor that allows for tissue homogenization. Also, it has a pre-inserted mesh that retains larger particles, removing them from the homogenized sample. The tube was then placed in the gentleMACS™ Dissociator (Miltenyi, Biotec) for the automated disruption of host cells. The homogenized sample containing the parasites was then passed through a column containing anion exchange preswollen microgranular diethylaminoethyl cellulose (DE52, Whatman). The sialylated TCT forms of the parasites were retained in the resin through
ionic interaction, while the ICA forms were recovered (Marques 2011).

**Compounds**

Compounds DDD86481, DDD85646, DDD87769, DDD99837, DDD100097, DDD99742, DDD100153 and DDD100144 were obtained from our collaborators Dr. Stephen Brand and Dr. Paul Wyatt (University of Dundee, UK). Patent WO2010/026365.

**In vitro proliferation assay**

Compounds DDD86481, DDD85646, DDD87769, DDD99837, DDD100097, DDD99742, DDD100153 and DDD100144 and the reference drug Benznidazol (BZ) were prepared 6X concentrated in 6% DMSO, to give a final concentration of 1X compound in 1% DMSO. 30 µL of the compounds were plated to give the final concentrations of 50, 10, 1, 0.1, 0.01 and 0.001 µM, while BZ was plated at a final concentration of 800, 400 and 40 µM. DMSO control wells contained 30 µL of 6% DMSO. Human osteocytes (U2OS cells) were diluted in Dulbeco’s Modified Eagle Medium (DMEM), 10% heat inactivated-fetal bovine serum (HI-FBS) to give a cell density of $2 \times 10^5$ cells/mL. *T. cruzi* trypomastigotes were resuspended in 10% HI-FBS, DMEM to a final density of $1.2 \times 10^7$. Cells and parasites were mixed a ratio of 4 volumes of cells plus 1 volume of parasites. 150 µL of the mixture were dispensed per well. Plates were incubated for 48 hours. After the incubation period, media was removed from the wells and 100 µL of 4% paraformaldehyde (PFA) were added. After 15 minutes of incubation at room temperature, PFA was removed and cells and parasites were stained with Draq5™ (Biostatus Limited) at a concentration of 5 µM diluted in PBS. The plates were then read
in an automated microscope.

**High-content imaging**

Image acquisition and analyses of the plates were carried out using the BD Pathway 855 high-resolution fluorescence bioimager system (BD Biosciences). Filter sets appropriate for the excitation and emission spectra of Draq5 were utilized. Images from four fields (2 x 2 montage) were acquired per well with a 20x objective. To perform the host cell segmentation and counting of parasites, the BD AttoVision™ v1.6.2 Sub Object analysis was used. Draq5 creates a background, staining the host cell and parasite nucleus, but defining the cytoplasm as well, determining this way the amount of parasites within each mammalian cell. The host cell nucleus was excluded by size difference. Raw data was imported into the BD™ Image Data Explorer (BD Biosciences) to determine the percentage of infected cells containing at least 1, at least 3 or at least 5 parasites.

**Cytotoxicity assay**

Compounds DDD86481, DDD85646, DDD87769, DDD99837, DDD100097, DDD99742, DDD100153 and DDD100144 and the reference drug Benznidazol (BZ) were prepared 6X concentrated in 6% DMSO, to give a final concentration of 1X compound in 1% DMSO. 30 µL of the compounds were plated to give the final concentrations of 50, 10, 1, 0.1 and 0.01 µM. DMSO control wells contained 30 µL of 6% DMSO, while wells for the negative control contained 1% hydrogen peroxide in media. U2OS cells were diluted in 10% HI-FBS, DMEM to give a cell density of 2x10^5 cells/mL, while ICA were
resuspended to a final density of $1 \times 10^7$ per well. Epis were resuspended in LIT to a density of $1 \times 10^6$ parasites per well. 150 µL of the suspension containing either cells or parasites were added per well, followed by a 48 hour-incubation for U2OS cells, 24 hours for ICA, 48 and 72 hours for Epis. After 47 hours of incubation for mammalian cells and 23 for parasites, a mixture of Propidium Iodide (Invitrogen) and Hoescht (Invitrogen) diluted in culture media (1 µg/mL each) was added to the plates. After one hour of incubation, plates were read in an automated microscope. In the case of the Epis, after 44 or 68 hours of incubation 18 µL of Alamar Blue (AbD Serotec) were added per well. After further incubation of 4 hours, the plate was read in a microplate fluorometer (Thermo Scientific) at excitation 560nm, emission 590 nm.

Determination of $IC_{50}$

$IC_{50}$ were determined using GraphPad software (GraphPad Software, Inc.) using a sigmoidal dose-response variable slope model with response values based on the total number of parasites or cells (normalized to span a range from 0 to 100%) plotted against the logarithm of compound concentration.

In-Gel Western Blot

$10^7$ parasites were incubated with or without 10 µM of the inhibitors DDD86481, DDD100097 and DDD100144 in 1 mL of 2% delipidated bovine serum albumin (BSA), DMEM at 37°C for ICA and TCT and at 28°C for Epis. After 6 hours, 100 µM of myristic acid azide (Life Technologies) (from 50 mM stock in DMSO) were added to the samples, and the same volume of DMSO was added to the negative control. Cells were
incubated for another 6 hours, for a 12-hour treatment with the inhibitors. Next, the parasites were washed three times with PBS and lysed in 1% SDS, Tris HCl, pH 8.0. “Click” reaction was performed between the myristic acid azide and Biotin Alkyne (Life Technologies) according to the manufacturer instructions using the Click-iT® Protein Reaction Buffer Kit (Life Technologies). Duplicate aliquots of lysates were ran in 10% SDS-PAGE followed by fixing (5% acetic acid, 50% isopropanol) for 15 min. Gels were then analyzed by in-gel western blot using IRDye® streptavidin (Licor) 1:7500 in 5% BSA, 0.05% SDS, 0.2% Tween in PBS, for 1 hour at room temperature. They were subsequently treated or not with 0.2 M NaOH in methanol for 1 hour at room temperature. Gels were scanned in an ODYSSEY® quantitative imagining system (Licor). Next, they were stained with Coomassie blue and scanned again to determine total protein levels.

\[^{35}S\] Methionine labeling

10^7 parasites were incubated with or without 10 μM of the inhibitors as described above. After 10 hours, cells were starved in methionine deficient media for 1 hour. Next, 20 μCi mL^-1 of \[^{35}S\] methionine (Perkin Elmer) were added to the samples for 1 hour incubation, for a 12-hour treatment with the inhibitors. Parasites were then washed three times with PBS and lysed in 1% SDS, Tris HCl, pH 8.0. Samples were ran in 4-12% gels (Exppeleon) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were dried and exposed to film. To determine total protein levels, membranes were stained with Ponceau S after exposure.
Metabolic labeling and fluorescence microscopy

10^7 parasites were incubated with or without 10 µM of the inhibitors DDD86481, DDD100097 and DDD100144 in 1 mL of 2% delipidated BSA-DMEM at 37°C for ICA and TCT and at 28°C for Epis. After 6 hours, 100 µM of myristic acid azide (from 50 mM stock in DMSO) were added to the samples, and the same volume of DMSO was added to the negative control. Parasites were further incubated for 6 hours for a total treatment of 12 hours with the inhibitors. Cells were washed with phosphate buffered saline (PBS) and deposited on a 96-well plate, followed by centrifugation at 3500 rpm for 10 min at room temperature. Supernatant was removed and cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature, then permeabilized with 0.1% Triton X-100 in PBS for 10 min. After permeabilization, cells were washed three times with 3% BSA in PBS. “Click” reaction between the myristic acid azide and Alexa Fluor 488® alkyne (Life Technologies) was performed according to the manufacturer instructions using the Click-iT® Cell Reaction Buffer Kit (Life Technologies). Parasite DNA was labeled with 4’,6-diamidino-2-phenylindole (DAPI) at 0.5 µg/mL. Samples were visualized using a LSM 700 Confocal Microscope (Zeiss) with a 63X oil objective lens. Images were acquired using the ZEN 2009 software (Zeiss).

Immunofluorescence Assay

10^7 parasites were incubated for 12 hours with or without 10 µM of the inhibitors DDD86481, DDD100097 and DDD100144 in DMEM, 10% HI-FBS, DMEM at 37°C for ICA and TCT and at 28°C for Epis. Parasites were fixed and permeabilized as described above. Wells were then blocked for 1 hour at room temperature with 3%
BSA-PBS. To obtain polyclonal anti-TcNMT, rabbit serum was rose against the TcNMT peptide RGDGNLHYYFYNWSYP (Biomatik). Parasites were incubated overnight with anti-TcNMT at 1:250 dilution in 3% BSA-PBS, followed by anti-rabbit IgG Alexa® 594 conjugated. Parasite DNA was then labeled with DAPI at 0.5 µg/mL. Samples were visualized and images acquired as described above.

Statistical analysis

Statistical significance for the in-cell western blot data was determined by Student’s t-test using GraphPad software (GraphPad Software Inc.). Differences were considered to be statistically significant up to p value <0.05.

Western Blot

10^7 parasites were incubated with or without 10 µM of the inhibitors DDD86481, DDD100097 and DDD100144 in 1 mL of 10% HI-FBS, DMEM at 37°C for ICA and TCT and at 28°C for Epis for 12 hours. Next, parasites were washed three times with PBS and lysed in 1% SDS, Tris HCl, pH 8.0. Samples were run in 10% SDS-PAGE followed by western blot probed with anti-TcNMT at dilution 1:1000 in 5% milk-PBS overnight. This was followed by incubation with anti-rabbit IgG-HRP conjugated. The membrane was next stripped with boiling stripping buffer (3% SDS, 0.3% β-mercaptoethanol) for 10 min, followed by 4 washes with 0.1% PBS-T. It was then blot again with anti-BiP at dilution 1:500 in 5% milk-PBS overnight, followed by anti-rabbit IgG HRP-conjugated.
3.3. Results

DDD compounds inhibit *T. cruzi* intracellular proliferation

Previous studies demonstrated the efficacy of the studied pyrazole sulphonamides as NMT inhibitors against *T. brucei* (Frearson, Brand et al. 2010; Brand, Cleghorn et al. 2012). Based on these studies, we tested selected compounds to determine their effects against the intracellular proliferation of *T. cruzi* on human osteocytes (U2OS cells). Draq5 (Biostatus Limited) stains nucleic acids; however, it creates a background defining the cytoplasm of the host cell. Therefore, it stains the nucleus of the host cell and the parasite with a higher intensity, while staining the cytoplasm of the mammalian cell with a fainter intensity, rendering the software able to distinguish one from the other. An example of the segmentation performed on the fluorescence bioimager is shown in Figure 3.1. Upon entering the cell, trypomastigotes differentiate into replicative amastigote forms and begin to divide. Approximately 96 hours after infection the parasite transforms again into trypomastigotes, finally rupturing the cell and releasing the parasites into the extracellular milieu (Epting, Coates et al. 2010). Since we are interested in the antiproliferative effects of the compounds, we incubated for 48 hours prior to fixation. At this time point, significant replication of the parasite is observed with little host cell disruption. To determine the percentage of infected cells, we performed a constriction by obtaining the number of cells containing at least one parasite (Figure 3.2.A). We did not see significant differences between the untreated wells and the wells treated with the compounds. To determine the
proliferation inside the mammalian cells, the number of cells containing at least 3 (Figure 3.2.B) or 5 (Figure 3.2.C) parasites was plotted. Compounds DDD86481, DDD100097 and DDD100144 showed inhibition of intracellular proliferation, with submicromolar IC$_{50}$ of 0.175 µM, 0.258 µM and 0.346 µM respectively (Table 3.1).
Figure 3.1. High-content imaging and data analysis.

Representative images of cells treated with the vehicle control (DMSO), untreated, treated with 800 µM Benznidazol or 10 µM DDD100144, stained with Draq5 (left panel). Artificial images created after segmentation on the fluorescence bioimager (right panel). Host cells are shown in blue, extracellular parasites in red and intracellular parasites in pink.
Figure 3.2 DDD compounds inhibit intracellular proliferation of *T. cruzi*.

The multiparametric data obtained on a cell-by-cell basis was analyzed to determine several parameters associated to infection of host cells by *T. cruzi* including the (A) percentage of cells infected with at least one parasite (percentage of infected cells), percentage of cells infected with (B) at least 3 or (C) at least 5 parasites (percentage of
cells in which the parasite proliferated).
Table 3.1. IC$_{50}$ values of DDD86481, DDD100097 and DDD100144.

<table>
<thead>
<tr>
<th>Compound</th>
<th>T. cruzi Proliferation Assay (µM)</th>
<th>T. cruzi Purified ICAs (µM)</th>
<th>U2OS Cells (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDD86481</td>
<td>0.175</td>
<td>1.83</td>
<td>12.82</td>
</tr>
<tr>
<td>DDD100097</td>
<td>0.258</td>
<td>0.059</td>
<td>11.38</td>
</tr>
<tr>
<td>DDD100144</td>
<td>0.346</td>
<td>0.017</td>
<td>11.94</td>
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</table>
DDD compounds are trypanocidal and not toxic to mammalian cells

These compounds were originally synthesized to target an extracellular parasite, namely *T. brucei*. Therefore, we wanted to determine if the cytotoxic effects against the intracellular parasite *T. cruzi* could be increased once the barrier of host cell membrane was not present. It is possible that a delivery method that will help the translocation of these compounds from the extracellular milieu into the host cell would increase the potency of the inhibitors against the parasite. To determine the effects directly on intracellular amastigotes (ICA), we purified them from the host cells and incubated them with the inhibitors for 24 hours. As expected, compounds DDD86481, DDD100097 and DDD100144 which were the most effective at inhibiting proliferation were also the most cytotoxic displaying an IC$_{50}$ of 1.83 µM, 0.059 µM and 0.017 µM, respectively (Figure 3.3 and Table 1). We were able to see a more potent effect of the inhibitors DDD100097 and DDD100144 when tested directly against ICA. This suggests that by improving the translocation of these two compounds into the mammalian host cell, the potency of the inhibitors against the intracellular parasites would be enhanced. DDD85646 had shown high efficacy against *T. brucei*, both *in vitro* and *in vivo*. Interestingly, in *T. cruzi* this compound displayed an IC$_{50}$ of 1.14 µM (Figure 3.2.C) in the proliferation assay and showed no significant trypanocidal activity against purified ICAs (Figure 3.3). The drug of reference, Benznidazole, at a concentration of 800 µM did not have a significant effect on parasite viability. To determine the cytotoxic effects of these compounds on mammalian cells, we performed a viability assay on U2OS cells. Using high content imaging we were able to differentiate dead cells by the uptake of propidium iodide. After
48 hours of treatment, compounds DDD86481, DDD100097, and DDD100144 showed toxicity only at 50 µM, while there was no significant effect at lower concentrations (Figure 3.4). Since almost complete inhibition of intracellular proliferation of the parasite was observed at concentrations as low as 10 µM there is a window of selectivity between human NMT and *T. cruzi* NMT (Table 1). Interestingly, the epimastigote form of the parasite was less susceptible to these compounds. No significant difference was observed on the viability of the parasite when increasing the length of the treatment from 48 to 72 hours. After 72 hours, DDD86481 did not have a significant effect on parasite viability, while DDD100097 and DDD100144 showed an IC$_{50}$ of 9.68 µM and 2.14 µM, respectively (Figure 3.5).
Figure 3.3. DDD compounds are trypanocidal against purified intracellular amastigotes. Total number of parasites in each well was counted to evaluate the cytotoxicity of the compounds against purified intracellular amastigotes.
**Figure 3.4.** DDD compounds are only toxic at high concentrations against mammalian cells.

Total number of cells in each well was counted to evaluate the cytotoxicity of the compounds against U2OS cells.
Figure 3.5. DDD compounds are less effective on the epimastigote form of the parasite. Parasite viability was quantitatively measured after (A) 48 and (B) 72 hours of treatment using a fluorometer at excitation 560nm, emission 590 nm.
Inhibitors act “on target”

To determine whether these inhibitors are acting on TcNMT, we labeled epimastigotes, amastigotes and trypomastigotes with myristic acid azide (Life Technologies), which had been previously incubated with or without 10 µM of the inhibitors. Detection of N-myristoylated proteins is typically performed by metabolic labeling with radioactive [\(^3\)H]-myristate. Alternatively, we used a bioorthogonal labeling method. In the first step, an azido-myristic acid analog was actively incorporated into the parasites. In the second step, the “click” reaction, the azide-modified proteins reacted with a chemoselective alkyne-biotin. After SDS-PAGE and in-gel western blot analysis with IRDye® 800CW Streptavidin (Licor), several putative N-myristoylated proteins were visualized. To confirm that these proteins were indeed N-myristoylated duplicate gels were treated with 0.2 M NaOH in methanol to remove any base-labile hydroxy- or thioester-linked myristic acid azide. Treatment with base prior to scanning showed no difference in the number of bands indicating that they are N-myristoylated proteins (data not shown).

Two bands, at 70 and 250 kDa, were present in all samples. They probably represent proteins that are endogenously biotynilated; such as the biotin-dependent carboxylases, 3-methylcrotonyl-CoA and acetyl-CoA carboxylase which have been extensively characterized in T. brucei (Vigueira and Paul 2011). Incubation of the parasites with 10 µM of DDD86481, DDD100097 and DDD100144 for 12 hours resulted in either a decreased intensity or complete loss of most of the bands in all the stages of the parasite (Figure 3.6). To determine whether these compounds specifically inhibited N-myristoylation, parasites from the three different stages of T. cruzi were labeled with
[\textsuperscript{35}S] methionine. Treatment with DDD86481, DDD100097 and DDD100144 had no effect on the incorporation of [\textsuperscript{35}S] methionine into proteins (Figure 3.7), indicating that the inhibitors do not affect protein translation. This data suggests that the DDD compounds specifically inhibit N-myristoylation in all \textit{T. cruzi} stages and that this is directly linked to inhibition of proliferation.
Figure 3.6. DDD compounds specifically inhibit TcNMT.

In-gel western blots of lysates of (A) Epis, (B) ICAs and (C) TCTs treated with or without 10 µM of the inhibitors, labeled with myristic acid azide followed by “click” reaction with biotin alkyne. IRDye® 800CW streptavidin was used to detect myristoylated proteins.
**Figure 3.7.** DDD compounds do not affect protein translation.

Fluorographs of PVDF membranes from lysates of (A) Epis, (B) ICAs and (C) TCTs treated with or without 10 µM of the inhibitors, labeled with $[^{35}\text{S}]$ methionine.
DDD compounds decrease the overall levels of myristic acid in epimastigotes

To determine if the overall cellular levels of myristic acid in treated parasites were affected, we labeled Epis, ICAs and TCTs with myristic acid azide, followed by “click” reaction with Alexa Fluor® 488 alkyne. Fluorescence microscopy showed a decrease in the intensity of the signal in Epis (Figure 3.8) treated with the three compounds after 12 hours relative to the untreated parasites. In the case of ICAs (Figure 3.9) only DDD100097 and DDD100144 affected the total levels of myristic acid. In TCTs there was no observable reduction in the amount of myristic acid (Figure 3.10). In order to determine the total levels of myristic acid quantitatively, we used in-cell western blot analysis. In brief, we labeled treated parasites with myristic acid azide, followed by “click” reaction with biotin alkyne. To detect the levels of myristic acid we used IRDye® 800CW Streptavidin. The streptavidin signal was normalized to the levels of BiP (binding protein), by using rabbit anti-BiP (Bangs, Uyetake et al. 1993) followed by detection with IRDye® 680RD anti-rabbit IgG. A significant reduction in the levels of total levels of myristic acid was observed in treated Epis (Figure 3.11). However, we were unable to see this effect in treated ICAs and TCTs.
Figure 3.8. Overall levels of myristic acid in epimastigotes treated with DDD compounds.

Fluorescence microscopy of Epis treated with 10 µM of the inhibitors and metabolically labeled with myristic acid azide were reacted with Alexa® 488 alkyne (green) after permeabilization, co-stained with DAPI (blue) to label the nucleus and kinetoplast. Scale bar, 5 µm.
**Figure 3.9.** Overall levels of myristic acid in amastigotes treated with DDD compounds. Fluorescence microscopy of ICAs treated with 10 µM of the inhibitors and metabolically labeled with myristic acid azide were reacted with Alexa® 488 alkyne (green) after permeabilization, co-stained with DAPI (blue) to label the nucleus and kinetoplast. Scale bar, 5 µm.
Figure 3.10. Overall levels of myristic acid in trypomastigotes treated with DDD compounds.

Fluorescence microscopy of TCTs treated with 10 µM of the inhibitors and metabolically labeled with myristic acid azide were reacted with Alexa® 488 alkyne (green) after permeabilization, co-stained with DAPI (blue) to label the nucleus and kinetoplast. Scale bar, 5 µm.
Figure 3.11. Quantitative analysis of overall levels of myristic acid in epimastigotes treated with DDD compounds.

In-cell western blot of Epis treated with 10 µM of the inhibitors for 12 hours and metabolically labeled with myristic acid azide, followed by “click reaction” with biotin alkyne. Myristic acid levels were normalized to the levels of BiP.
Epimastigotes treated with DDD compounds overexpress NMT

To investigate the expression and cellular localization of TcNMT in parasites treated with the compounds, Epis, ICAs and TCTs were analyzed by immunofluorescence after 12 h of treatment with 10 µM of DDD86481, DDD100097 and DDD100144. Anti-TcNMT was obtained from rabbit sera rose against the TcNMT peptide RGDGNLHYFYNSYP (Biomatik). Anti-TcNMT labeling in treated parasites showed the same cellular localization as that seen in untreated parasites. Nonetheless, Epis treated with the three different inhibitors showed an increased signal, suggesting overexpression of this enzyme (Figure 3.12). In ICAs the same effect was observed with all the inhibitors (Figure 3.13), while only TCTs treated with DDD86481 and DDD100097 showed increased signal whereas DDD100144 did not affect TcNMT expression (Figure 3.14). To confirm these results in a more quantitatively manner, we used western blot analysis on lysates from parasites treated with or without the inhibitors. TcNMT in lysates from wild type parasites could not be detected by western blotting, probably due to low expression in all stages of T. cruzi. However, treated Epis showed an overexpression of TcNMT that was detectable by western blotting (Figure 3.15). We were unable to see the same phenomenon in lysates from ICAs and TCTs pre-incubated with the DDD compounds. These data suggest that the slight increment in TcNMT signal observed in immunofluorescence analysis in treated ICAs and TCTs might be not significant. Moreover, these observations may explain the increased resistance of Epis to the inhibitors (Figure 3.5). Even though we still see a reduction in protein N-myristoylation in Epis (Figure 3.6), the overexpression of TcNMT might be
enough to compensate for the inhibitory effects of the compounds, rendering these parasites less susceptible to the treatment. Encouragingly, these data further confirms the “on-target” effect of these compounds.
Figure 3.12. TcNMT expression in epimastigotes treated with DDD compounds.

Immunofluorescence microscopy of Epis treated with 10 µM of the inhibitors. Cells are shown as viewed under phase contrast, visualized for fluorescence with anti-TcNMT (red), co-stained with DAPI to reveal positions of the nucleus and kinetoplast (blue). Scale bar, 5 µm.
Figure 3.13 TcNMT expression in amastigotes treated with DDD compounds.

Immunofluorescence microscopy ICAs treated with 10 µM of the inhibitors. Cells are shown as viewed under phase contrast, visualized for fluorescence with anti-TcNMT (red), co-stained with DAPI to reveal positions of the nucleus and kinetoplast (blue). Scale bar, 2 µm.
**Figure 3.14** TcNMT expression in trypomastigotes treated with DDD compounds.

Immunofluorescence microscopy TCTs treated with 10 µM of the inhibitors. Cells are shown as viewed under phase contrast, visualized for fluorescence with anti-TcNMT (red), co-stained with DAPI to reveal positions of the nucleus and kinetoplast (blue). Scale bar, 2 µm.
Figure 3.15. TcNMT is overexpressed in epimastigotes treated with DDD compounds. Epis were treated with 12 hours with or without 10 µM of the inhibitors. Levels of NMT expression were confirmed by western blotting using anti-TcNMT. BiP (binding protein) was used as a loading control.
Chapter 4: Discussion and Final Conclusions

Despite the impressive advances in understanding the protozoan parasite *T. cruzi*, Chagas disease continues to cause significant morbidity and mortality. Most of the drugs available to threat this disease are decades old, limited in efficacy and present severe side effects. Moreover, there are no vaccines available, which has classified Chagas disease as a neglected tropical disease. There is an urgent need for new chemotherapeutic targets and approaches aiming at the development of novel compounds against this parasite. Here, we present the first evidence that validates *T. cruzi* N-myristoyltransferase as a potential chemotherapeutic target.

*N*-myristoyltransferase has been intensively studied as a drug target for fungal and parasitic infections. In *T. cruzi* over one hundred proteins have been predicted to be *N*-myristoylated (Mills, Price et al. 2007), suggesting that TcNMT inhibition would have pleotropic effects in the physiology of this parasite. For example, the flagellar calcium binding protein (FCaBP), a protein involved in the transduction of a flagellum specific calcium modulated signal, flagellar biosynthesis and movement, is both myristoylated and palmitoylated. These acyl modifications are essential for flagellar association. Alanine substitution at the glycine residue in the second position of the coding sequence renders this protein cytosolic (Godsel and Engman 1999). Another protein, namely phosphoinositide-specific phospholipase C (PI-PLC), has been experimentally validated to be *N*-myristoylated. This modification is necessary for membrane targeting and for stimulation of differentiation of the infective trypomastigote into the amastigote form (Okura, Fang et al. 2005). Despite the obvious importance of this lipid modification in *T.
cruzi, no effort has been taken in exploring TcNMT as a potential drug target.

This work provides a promising starting point for the development of TcNMT as a target for urgently needed antichagasic agents. The first part of this work focuses on the expression and localization of TcNMT. Sequence analysis has shown conservation of residues predicted to be essential for NMT activity. Interestingly, two ~30 aminoacid insertions are found in TcNMT, suggesting additional secondary and tertiary structures that could affect TcNMT activity relative to human NMT. These differences between parasite and human NMT can be exploited in the development of specific TcNMT inhibitors. NMTs from L. major and T. brucei have been shown to partition between membrane and cytosolic fractions (Price, Menon et al. 2003; Panethymitaki, Bowyer et al. 2006). Here we show by colocalization with BiP that TcNMT is at least partially associated to the ER. Moreover, we provide evidence that NMT is constitutively expressed, and most importantly present in those stages of T. cruzi that cause disease in mammals, further highlighting the importance of this enzyme.

Furthermore, we have standardized the heterologous expression and purification of recombinant TcNMT, which will allow for future kinetic analyses, and for the development of in vitro inhibition assays. Recently, a “piggyback” approach, which involves screening of antifungal NMT inhibitors, has been used in the development of drugs against protozoan parasites (Bowyer, Tate et al. 2008). In L. major, T. brucei and P. falciparum (Panethymitaki, Bowyer et al. 2006; Bowyer, Gunaratne et al. 2007), a scintillation proximity assay using recombinant NMTs was used for high-throughput screening of these antifungal NMT inhibitors. The same approach can be used in the case of T. cruzi, where libraries of compounds can be screened against recombinant
Traditionally, detection of fatty-acylation has been performed by metabolic labeling with radioactive fatty acids followed by visualization using autoradiography (Zeidman, Jackson et al. 2009). Although effective, radioactivity often requires days to weeks to visualize lipid-modified proteins. Recently, chemical reporters that enable rapid detection of N-myristoylated proteins using bioorthogonal labeling methods (“click chemistry”) have been developed (Charron, Zhang et al. 2009) and are now commercially available (Life Technologies). Despite the availability of these reporters, \[^3\text{H}\] myristic acid continues to be the choice in the study of N-myristoyltransferase in protozoan parasites. Here, we have validated and standardized the metabolic labeling of trypanosomes with azido-myristic acid analogs, followed by chemoselective ligation with the corresponding hapten- or fluorophore-alkyne. This work presents, to our knowledge, the first alternative, non-radioactive method to study N-myristoylation in kinetoplastids.

The second part of this work focuses on the evaluation of NMT inhibitors as anti-\textit{T. cruzi} agents. Screening of a 62,000 diversity-based compound library against \textit{T. brucei} NMT, led to the discovery of potent inhibitors that cured trypanosomiasis \textit{in vivo} (Frearson, Brand et al. 2010). In this study we tested eight of this inhibitors against the intracellular parasite \textit{T. cruzi}. We have presented evidence of strong inhibition of intracellular amastigote proliferation particularly with compounds DDD86481, DDD100097 and DDD100144. Importantly, they did not show cytotoxic effects against mammalian cells at effective trypanocidal concentrations. Interestingly, DDD85646, which showed potent \textit{in vitro} and \textit{in vivo} activity against \textit{T. brucei}, showed no
trypanocidal activity against purified ICAs. In addition, we have demonstrated the “on target” effect of these compounds by metabolic labeling with myristic acid azide. In-gel western blots of lysates from parasites showed a significant decrease in protein N-myristoylation relative to the untreated controls. Strikingly, Epis were less susceptible to these compounds, which might be explained by the TcNMT overexpression observed in this stage of the parasite. This overexpression suggests a compensation mechanism for the inhibition of TcNMT, confirming the specificity of the inhibitors. Overall, it is likely that the effects of the tested compounds are a result of several downstream events, as this enzyme has over 100 putative substrates (Mills, Price et al. 2007). The lead compounds hold great potential to be explored as antichagasic agents. Therefore, additional studies will be carried out to further evaluate the in vivo effects of DDD86481, DDD100097 and DDD100144 in the murine model of Chagas disease.

In conclusion, the results from this dissertation provide an insight in a novel potential chemotherapeutic target in this parasite. Further characterization of this enzyme will be aid by the techniques standardized in this work. Here, we have validated TcNMT as a potential drug target in Chagas disease. Importantly, the tested compounds demonstrated significant and specific anti-proliferative activity and hold great potential to be explored as anti-\textit{T. cruzi} agents. The knowledge accumulated through these studies may eventually result in the development of improved chemotherapeutics against Chagas disease.
4.1 Acknowledgments

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References


## Appendix

### List of abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BiP</td>
<td>Binding protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BZ</td>
<td>Benznidazol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’6-diamidino-2-phenylindole</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dmethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>Epi</td>
<td>Epimastigote</td>
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<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<td>HI-FBS</td>
<td>Heat-inactivated fetal bovine serum</td>
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<td>IC_{50}</td>
<td>Half maximal inhibitory concentration</td>
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<td>ICA</td>
<td>Intracellular amastigotes</td>
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<td>IFA</td>
<td>Immunofluorescence assay</td>
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<td>IPTG</td>
<td>Isopropyl β-D-thiogalactoside</td>
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<td>LIT</td>
<td>Liver infusion tryptose</td>
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<tr>
<td>LLC-MK2</td>
<td>Rhesus monkey kidney epithelial cells</td>
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<tr>
<td>LmNMT</td>
<td><em>Leishmania major</em> N-myristoyltransferase</td>
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<tr>
<td>NMT</td>
<td>N-miristoyltranferase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrilamide gel electrophoresis</td>
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<tr>
<td>TbNMT</td>
<td>Trypanosoma brucei N-myristoyltransferase</td>
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<tr>
<td>TcNMT</td>
<td>Trypanosoma cruzi N-myrsitoyltransferase</td>
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<tr>
<td>TCT</td>
<td>Tissue culture cell-derived trypomastigotes</td>
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<tr>
<td>U2OS</td>
<td>Human osteocytes</td>
</tr>
</tbody>
</table>
List of publications and manuscripts


Curriculum vitae

Linda Herrera was born in Morelia, Michoacan, Mexico. The oldest of two children of Enrique Herrera and Yolanda Rodriguez, she received her Bachelor of Science degree in Microbiology from The University of Texas at El Paso in December 2007. She was admitted into the PhD program on January 2008 also in The University of Texas at El Paso. While pursuing her degree, she worked as a research technician and was also a teacher assistant in charge of different laboratories. From 2008 to 2013, she has taught Human Anatomy, Organismal Biology, Immunology and General Microbiology.

She has been working under the supervision of Dr. Rosa A. Maldonado. Her research has focused on fatty acid metabolism, particularly in the validation of N-myrsitoyltransferase as a chemotherapeutic target in Chagas disease. Throughout her graduate studies, she has been recipient of several awards, including numerous travel awards to attend scientific conferences. She was also accepted into the highly prestigious program “Biology of Parasitism” in 2010. She spent the summer in Woodshole, MA working side-by-side at the benchtop with leaders in the parasitology field.

In addition, she has presented her research in several meetings throughout the country; including, the Keystone Symposia: Drug Discovery for Protozoan Parasites in 2009 and 2012; and the Gordon Research Conference: Biology of Host-Parasite Interactions in 2012. She also received an oral presentation award and a poster.
presentation award at The Annual Meeting of the Rio Grande Branch of the American Society for Microbiology in 2008 and 2011, respectively.

She has already published one per-reviewed paper as co-author and soon will publish one as first author and another one as co-author. She was accepted into Texas A&M College of medicine, which she will be attending after graduation. Her long-term career goal is to become an independent clinical-research physician focused on infectious diseases.

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