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The *Trypanosoma brucei*  
sphingolipid synthase, an essential  
enzyme and a drug target

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Ssu-Ying Pan

MSc

2009

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## Abstract

Sphingolipids are important structural components of eukaryotic membranes, in addition they and their precursors are involved in signal transduction processes. In eukaryotes the biosynthetic pathway of sphingolipid biosynthesis is largely conserved. However, whilst mammals produce sphingomyelin (SM) as the major phosphosphingolipid using the enzyme SM synthase, plants and many pathogenic fungi and protozoa synthesize inositol phosphorylceramide (IPC) by IPC synthase. This enzyme is a target for anti-fungal drugs and functional orthologues have recently been identified in the insect vector-borne pathogenic kinetoplastid protozoa. These parasites are responsible for a range of neglected diseases and *Trypanosoma brucei* are the cause human African trypanosomiasis in many regions of Africa. The available treatments for this disease are limited, often demonstrate severe side-effects and drug resistance is increasing. *T. brucei* sphingolipid synthase (*TbSLS*), a functional orthologue of the yeast IPC synthase, may be a target for novel anti-protozoals drugs. Here I show that this enzyme functions as an IPC synthase both *in vitro* and *ex vivo*. Furthermore, the *TbSLS* is essential for parasite growth and can be inhibited *in vitro* by a known anti-fungal. Perhaps most importantly this drug demonstrates rapid trypanocidal activity against bloodstream form parasites. Thus *TbSLS* is a promising drug target.

## **Acknowledgements**

I would like to thank my parents, all of my teachers, and my supervisor (Dr. Paul W. Denny). I would appreciate Dr. Denny as my supervisor for training and teaching me a lot. Thanks to all of my colleagues, Dr Lindsay, Dr Fiona, Dr. Steve, Ahmed, Bing, John, Jongwoo, Li Zhang, Nilu, Tamarai, and Victor who taught me many experimental techniques and knowledge. I can't finish this report without your help and support.

## **Declaration**

I declare that all the work in this report was made by my own, and that has not been used in a thesis, dissertation or a report submitted to this Durham University for a degree, diploma or other qualification.

Signed : \_\_\_\_\_

Ssuying, Pan

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## Abbreviations

AbA	Aureobasidin A
BSF	Bloodstream form
BBB	blood-brain barrier
CATT	Card agglutination trypanosomiasis test
CHAPS	2.5% 3-[3-(cholamidopropyl) dimethylammonio]-1-propane sulfonate
CHO	Chinese hamster ovary
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computerized tomographic
CYC	Cycloheximide
DMFO	Eflomithine
EEG	Electroencephalogram
ER	Endoplasmic reticulum
GPI	Glycosylphosphatidylinositol
GSL	Glycosphingolipid
HAPT1	High-affinity pentamidine transporter 1
HPTLC	High performance thin layer chromatography
IPC	Inositol phosphorylceramide
LAMP	Loop-mediated isothermal amplification
LAPT1	Low-affinity pentamidine transporter 1
MR	Magnetic resonance
MYR	Myriocin
ODC	Ornithine decarboxylase
ORF	Open reading frame
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PSL	Phosphosphingolipid
PTRE	Post-treatment reactive encephalopathy
RNAi	RNA interference

REM	Rapid eye movement
SLS	Sphingolipid synthase
SM	Sphingomyelin
SMB	Single marker bloodstream-form
SPT	Serine palmitoyltransferase
SRA gene	Serum resistance associated gene
TDR	Tropical disease research
<i>TbSLS</i>	<i>T. brucei</i> sphingolipid synthase
TLF	Trypanosome lytic factor
VSG	Variable surface glycoprotein

# **Chapter 1:**

# **Introduction**

# Chapter 1: Introduction

## 1.1 *Trypanosoma brucei* and disease

### 1.1.1 General information

*Trypanosoma brucei* (Kinetoplastae, Trypanosomatidae) are unicellular eukaryotes that infect both human and economically important animals causing African sleeping sickness in sub-Saharan African (Raper et al., 2002). This tsetse fly-borne disease is classified as a tropical disease research (TDR) category 1 resurgent disease by the World Health Organization (WHO; Barrett et al., 2003).

According to the WHO, the people of 36 sub-Saharan countries are threatened by this disease and 300,000 new cases are reported every year (Masocha et al., 2007). It is estimated that 50000 people died following infection by *T. brucei* each year. (Fairlamb, 2003) Around 1 million people died from African sleeping sickness disease between 1896 and 1908 (Seed, 2001). However, during the 1950s, this disease was controlled using DDT to kill the vector and the progress of medical technology (Seed, 2001). Therefore, the number of cases decreased rapidly between 1949 and 1965 (figure 1.1). However, in the modern era, African sleeping sickness is out of control because of, amongst other things, war and the associated mass migration of people (Kennedy, 2004).

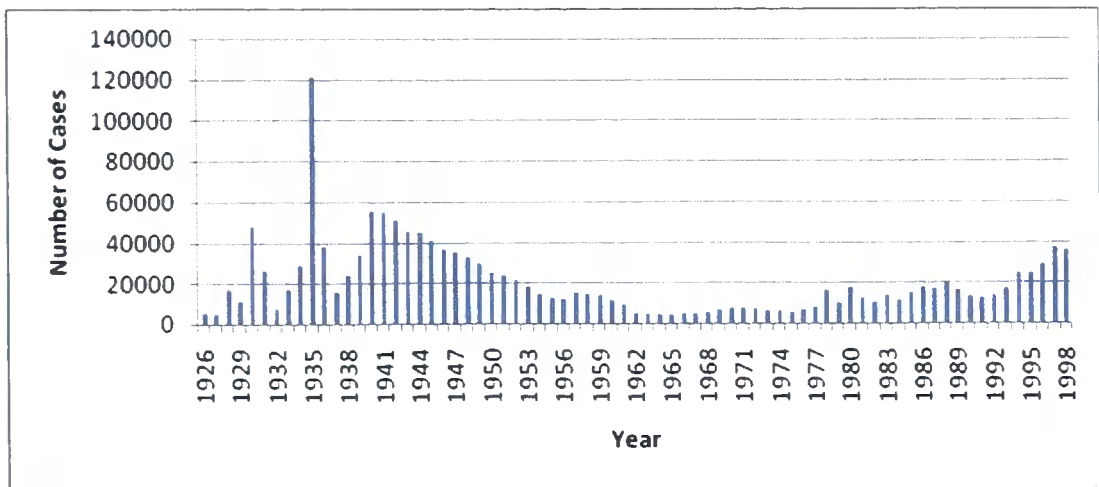


Figure 1.1

Newly registered cases of sleeping sickness, annually reported to WHO.

(<http://www.who.int/wer/2006/wer8108.pdf>)

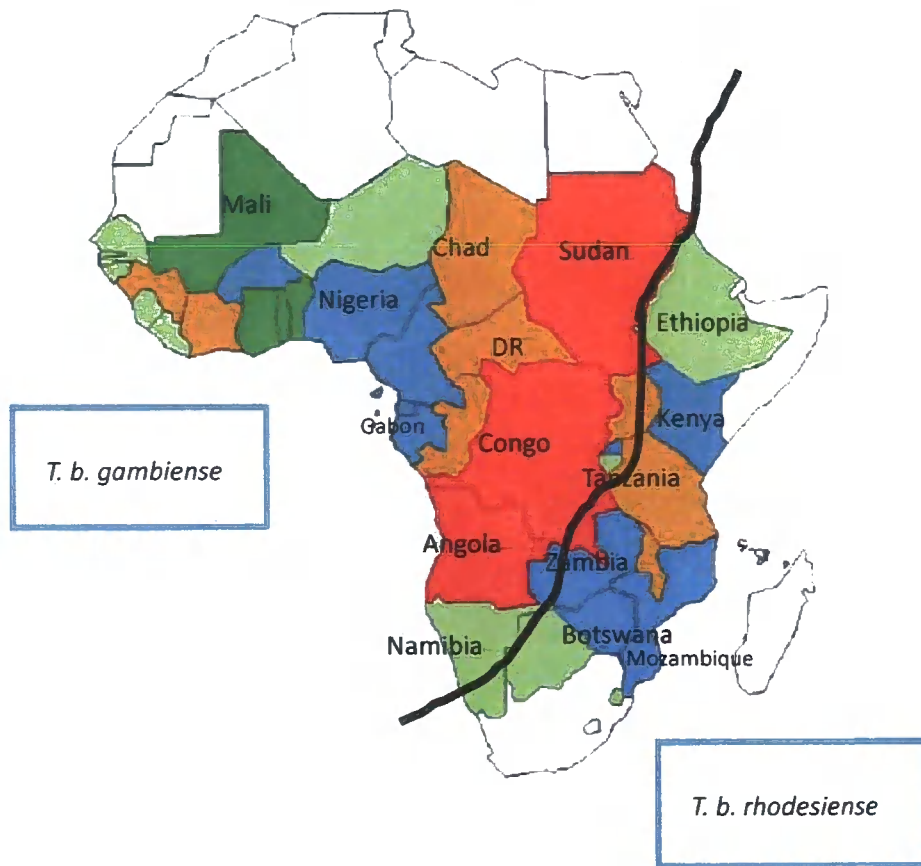


Figure 1.2

Human African trypanosomiasis endemicity in Africa (Barret et al., 2007).

Red: more than 1000 cases were reported per year. Orange: About 50 to 1000 cases were reported per year. Blue: less than 50 cases were reported per year.

Green: currently report no case of HAT.

There are two subspecies of African trypanosome that cause disease in humans: *T. b. gambiense* and *T. b. rhodesiense*. *T. b. brucei* causes Ngana in cattle, an economically important disease in sub-Saharan Africa, but cannot infect human because the parasite is lysed by components of the high-density lipoprotein fraction of human blood (Rifkin, 1978).

*T. b. gambiense* which causes West African Trypanosomiasis can be found in central and western African countries such as Sudan, Angola and Gabon (figure 1.2). *T. b. rhodesiense* which causes East African Trypanosomiasis can be found in central and eastern African countries such as Kenya, Tanzania and Malawi (figure 1.2, <http://www.cdc.gov/>).

Ninety percentage of African sleeping sickness cases are caused by *T. b. gambiense* and lead to a chronic infection (Barrett et al., 2003). Following a tsetse fly bite, the incubation period can be several months or years and when this disease is detected, the patient's central nervous system may already be damaged and the condition can become very dangerous. Other 10% of cases are caused by *T. b. rhodesiense* and lead to an acute infection (Parry et al., 2003). The appearance of symptoms can be few weeks or months after infection and the parasites invade can the central nervous system very rapidly (<http://www.cdc.gov/>).



### 1.1.2 *T. brucei* life cycle

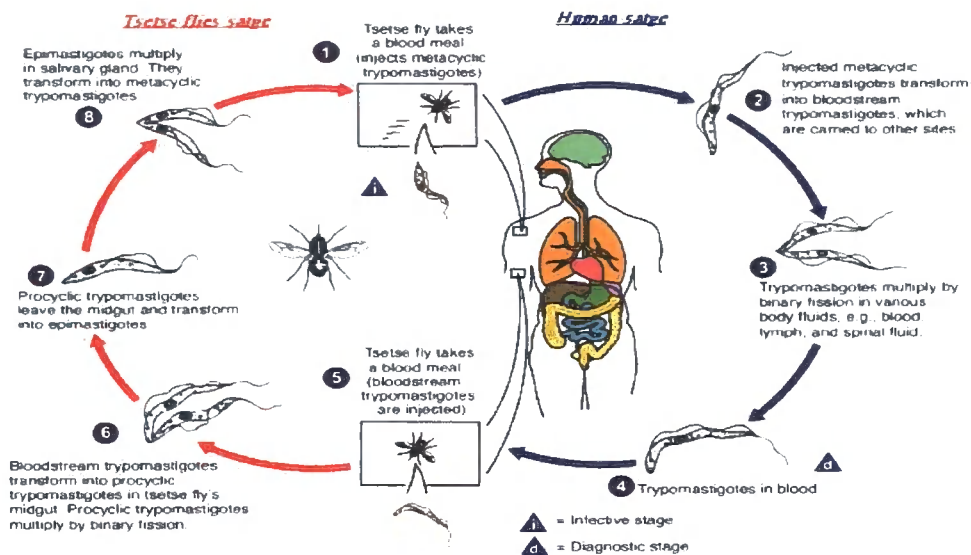


Figure 1.3

*T. brucei* life cycle from CDC (Kennedy, 2004).

The transmission of this trypanosome is caused by the bite of tsetse fly (*Glossina spp.*) which can be found in the grass near lakes or rivers, and in the forests and savannah of Africa (Parry et al., 2003).

After the fly bites a human (step 1), metacyclic trypanosomes transform into bloodstream form and start to transform the metacyclic variable surface glycoprotein (VSG) into the first bloodstream form VSGs at the site of inoculation (step 2, Donelson et al., 1985). This pathogenic form proliferates in the bloodstream (step 3) and can subsequently enter the lymph node and spinal fluid. At this stage, parasites can also be transmitted to non-infected Tsetse flies (step 5, <http://www.cdc.gov/>) where they transform to the procyclic form, losing the VSG coat, and replicate in the mid-gut of the vector (step 6). After 3 weeks, the procyclic forms migrate to the salivary (step 7) and transform into the metacyclic form which reacquires the VSG coat (step 8, Donelson et al., 1985). In addition, some reports showed that this parasite could also infect the foetus via the placenta and also be transmitted by contaminated needles (WHO, 2005).

### 1.1.3 Clinical features

Five days after infection, chancre may occur, especially in *T. b. rhodesiense* infection, at the site of the tsetse bite (figure 1.4; Barrett et al., 2003, Parry et al., 2003). This symptom is caused by the inflammatory response to the Trypanosomes (Naessens et al., 2003). Subsequently, there are two stages of African sleeping sickness: the haemolympathic stage and the encephalitic stage.

*Haemolympathic stage.* After one to three weeks, symptoms are nonspecific. The parasites escape from human adaptive immune response by antigenic variation via VSG which prevents trypanosome lysis by complement alternative pathway (Vincendeau and Bouteille, 2006, Mäser et al., 2003) and cause fever, malaise, arthralgia, weakness and weight loss (Sternberg, 2004). Many organs are infected by the bloodstream form trypanosomes at this stage, including the liver, skin, eye and cardiovascular system (Barrett et al., 2003). This plethora of symptoms can make initial diagnosis difficult; for example, patients are often misdiagnosed as suffering from malaria and so given anti-malarials (Parry et al., 2003, Kennedy, 2006). In *T. b. gambiense* infection, the major symptom is the enlargement of lymph nodes, especially in the triangle of the neck (Parry et al., 2003).

*Encephalitic stage.* At this stage, the parasites cross the blood-brain barrier are detectable in the cerebrospinal fluid (CSF) and cause damage to the central nervous system (Enanga et al., 2002). How the parasites cross blood-brain barrier is still unknown, however, it is assumed that *T. brucei* enter the central nervous system (CNS) by an active process (penetration) similar to white blood cells (Masocha et al., 2007). Again, it is difficult to diagnose the onset of this stage without examining the CSF for the presence of parasites. Patients' could change behavior becoming agitated and apathetic with severe headache, perhaps, losing the ability to eat and drink (Parry et al.,

2003). Untreated, patients fall into a coma and die (Sternberg, 2004).



Figure 1.4.  
Trypanosomal chancre on shoulder.

#### 1.1.4 Immune response

As well as facilitating immune evasion through antigenic variation, VSG also plays an important role in the human immune response to the trypanosomes. A trypanosome cell is covered with  $10^8$  molecules VSG attached to its surface membrane by a glycosylphosphatidylinositol (GPI) anchor. Here they act as the immune-dominant antigen activating both  $T_h$  and B cells (Mansfield, 1994). The GPI-moiety of soluble VSG released from the parasite directly leads to the over-activation of macrophages, which then produce  $TNF-\alpha$  after stimulation with  $IFN-\gamma$  probably produced from  $CD4+$  T cells.  $TNF-\alpha$  subsequently induces  $CD8+$  T cells to produce high levels of  $IFN-\gamma$  leading to a decrease in the formation of IL-2 and IL-2 receptors thus impairing the proliferative T cell response, causing immune-suppression and avoiding parasite elimination (Sternberg, 2004; Zambrano-Villa et al., 2002). In addition, increasing levels of IL-10 were observed in the CSF at the late stage of *T. brucei* infection related to the inflammatory response and the effects of NO synthesis (MacLean et al., 2001, Vincenedau and Bouteille, 2006).

The cattle parasite, *T. b. brucei*, is unable to infect humans due to the presence of trypanosome lytic factor (TLF) in sera (Raper et al., 2001). Unlike *T. b. brucei*, *T. b. rhodesiense* has a serum resistance associated (SRA) gene which encodes a protein protecting the parasites against TLF-mediated lysis and so avoids clearance from the bloodstream. However, *T. b. gambiense* lacks the SRA gene and is resistant to TLF via an unknown mechanism (Gibson, 2002).

In a further immune evasion strategy, trypanosomes produce a gp63-like protein similar to gp63 from *Leishmania* (Donelson et al., 1998), which is believed to protect bloodstream form parasites from complement-mediated lysis (Zambrano-Villa et al., 2002).

#### 1.1.5 Diagnosis

African sleeping sickness cannot be easily diagnosed due to the non-specific symptoms and multiple tests are often necessary (Kennedy, 2004). At the first, haemolymphatic stage, of the disease parasites can be detected in blood or infected tissue, such as lymph nodes, by staining and light microscopy (figure 1.5 and figure 1.6, Kennedy, 2004). However, this method is often initially invalid for *T. b. gambiense* as the infection is chronic and parasites appear at the peripheral rather than blood circulatory system (Kennedy, 2004). Under these circumstances, the antibody-detecting, card agglutination trypanosomiasis test (CATT) is the easiest and simplest way to reach a diagnosis (WHO, 2005). No infection is diagnosed when CATT is negative or the titre is less than 1:4, a positive diagnosis is reached when CATT is positive and the titre is more than 1:16. Following a positive CATT, it is necessary to check blood smears to confirm the presence of parasites before initiating treatment (Chappuis et al., 2005).

At the second, encephalitic stage, CSF extracted by lumbar puncture can allow the parasites to be detected directly. (Parry et al., 2003) An increase in the white blood cell count to more than 5/ $\mu$ l is also indicative and is a rise in IgM levels, as measured by a latex agglutination assay (Lejon et al., 2002). PCR of CSF can be an additional and sensitive diagnostic tool (Solano et al., 2002), however, its expense negates its use in many endemic countries. As an alternative, loop-mediated isothermal amplification (LAMP), in which DNA is amplified under isothermal (constant temperature) conditions of around 65°C, has been used negating the need for the costly thermocyclers required for PCR (Kuboki et al., 2003).

Neither computerized tomographic (CT), magnetic resonance (MR) nor electroencephalogram (EEG) showed any neurological abnormalities at this disease stage (Atouguia and Kennedy, 2000). However, the variation of brain activity indicated by rapid eye movement (REM) may be informative (Buguet et al., 2005).

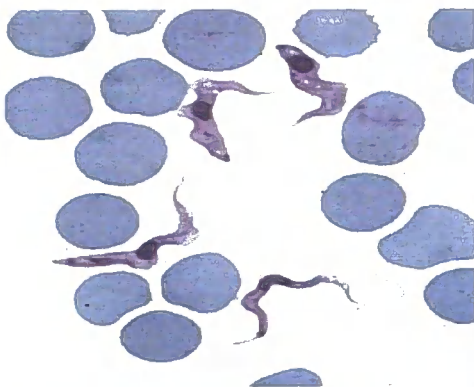


Figure 1.5  
*T. brucei* in human blood; thin film, Giemsa stain (Parry et al., 2003).



Figure 1.6  
Colored scanning electron micrograph of *T. brucei* in human blood (Kennedy, 2004).

There are four main drugs which have been used in the different stages in the disease: Suramin, Pentamidine, Melarsoprol and Eflomithine (DFMO). Three of them were established as treatments more than 50 years ago, with DFMO appearing more than 25 years ago (table 1.1, Vincendeau and Bouteille, 2006).

Suramin which is a water soluble colourless, polyanic sulfonated naphthylamine was introduced in the 1920s (Fairlamb, 2003). This drug, whose mechanism of action is unknown, is used to treat stage 1 *T. b. rhodesiense* infection but cannot efficiently penetrate the human blood-brain barrier (BBB) and so is ineffective in stage 2 (Fairlamb, 2003). Suramin is believed to inhibit topoisomerase II and interfere with white blood cell signaling in animal cells (Bojanowski et al., 1992). The side effects these interactions appear to cause include collapse, nausea, vomiting and shock. More seriously, latent kidney damage can occur leading to, for example, haemolytic anaemia which can prove fatal (Fairlamb, 2003). Although some cases showed that this drug may threaten the patient's life, it remains a first line treatment for early phase *T. b. rhodesiense* infections (Parry et al., 2003).

Pentamidine, which is an aromatic diamidine, was introduced in the 1940s (Fairlamb, 2003). Again, this drug cannot penetrate human BBB and therefore is only effective against stage 1 African sleeping sickness. Pentamidine enters the parasites via the P2 aminopurine permease which also transports melarsoprol (see below, Carter et al., 1995). In addition, pentamidine can be transported by high-affinity pentamidine transporter 1 (HAPT1) and a low-affinity pentamidine transporter 1 (LAPT1). Therefore, trypanosomes resistant to melarsoprol are still inhibited by pentamidine (de Koning, 2001). However, pentamidine is only used as a frontline treatment against *T. b. gambiense* as it is less reliable in the treatment of *T. b. rhodesiense* infections, where it is only used if suramin is not tolerated (Parry et al., 2003; Pépin and Milord,

1994). Pentamidine may cause organ damage of the liver, kidney and pancreas (Fairlamb, 2003). In addition, intravenous injection may lead to hypotensive reaction and therefore, this drug is administered intramuscularly (Fairlamb, 2003). The mechanism of action of pentamidine involves the inhibition of the *T. brucei* topoisomerase and so parasite replication (Shapiro and Englund, 1990).

Melarsoprol was developed in 1949 (Kennedy, 2004). The use of this drug is very extensive in the treatment of both *T. b. rhodesiense* and *T. b. gambiense* stage 2 infections. Melarsoprol is virtually insoluble in water, and is administered intravenously. (Fairlamb, 2003) It is known to inhibit the glycolytic enzyme, phosphogluconate dehydrogenase (Van Schaftingen et al., 1987, Hanau et al., 1996) and kills the parasite very quickly. However, it is also a very toxic and dangerous treatment (Parry et al., 2003). Side effects include vomiting, abdominal colic, arthralgia and thrombophlebitis. Furthermore, 5-10% patients suffer severe post-treatment reactive encephalopathy (PTRE) which is often fatal (Fairlamb, 2003, Pépin and Milord, 1994). Moreover, drug resistance caused by the disablement of the P2 aminopurine permease transporter is increasing (Delespaux and Koning, 2007). Melarsoprol resistance has now been found in the north west of Angola, north west of Uganda and south Sudan (Brun et al., 2001).

Eflomithine (Difluoromethylornithine; DFMO) was developed as an anti-tumor drug (Tabor and Tabor, 1984, Delespaux and de Koning, 2007) has been employed in the treatment of African sleeping sickness from 1981 (Kennedy, 2004). DFMO is an inhibitor of the first step of polyamide synthesis: ornithine decarboxylase (ODC) which catalyses the formation of putrescine from ornithine. The reason for its efficacy against African trypanosomes is unclear given that DFMO also inhibits the mammalian ODC. However, differences in the turnover rate of ODC in mammals and parasites may

provide an explanation (Heby, 2003). Similarly, the high turnover rate of ODC in *T. b. rhodesiense* compared with *T. b. gambiense* may explain why the former is insensitive to DFMO and the drug is only used to treat the late stage of infection with *T. b. gambiense* (Iten et al., 1997). In infection with this subspecies, DFMO has a greater effect on *T. b. gambiense* infection than melarsoprol, in addition it demonstrates much less toxicity and can be taken orally (Fairlamb, 2003). However, the high cost of this drug restricts its availability to patients with African sleeping sickness (Parry et al., 2003).

Nifurtimox was developed in 1977 and is used to treat Chagas disease, which is caused by American trypanosomes (Delespaux and de Koning, 2007). Nifurtimox maybe combined with DFMO to treat African sleeping sickness when melarsoprol or DFMO treatment was fails (Bray et al., 1994, Barrett et al., 2007). However, the efficiency of this drug is still under evaluation (Kennedy, 2004).

Following drug treatment, patients should receive follow up examinations every six months for at least two years, something very challenging in the endemic environments (Kennedy, 2004). The variation of VSG means a viable vaccine (the ideal situation) is unlikely to be developed (Matovu et al., 2003). This together with increasing drug resistance and the severe side effects (including death) of existing treatments mean it is necessary to urgently develop new drugs to treat the African sleeping sickness (Mäser et al., 2003).



<b>Drug</b>	<b>Spectrum</b>	<b>Indication</b>	<b>Year of first use</b>	<b>Route of Administration</b>
<b>Suramin</b>	<i>T. b. rhodesiense</i>	Stage 1	1920s	i.v.
<b>Pentamidine</b>	<i>T. b. gambiense</i>	Stage 1	1940	i.m.
<b>Melarsoprol (Mel B)</b>	<i>T. b. rhodesiense</i> <i>T. b. gambiense</i>	Stage 2	1949	i.v.
<b>Eflornithine (DFMO)</b>	<i>T. b. gambiense</i>	Stage 2	1981	i.v.

Table 1.1

Drugs currently used for the treatment of Human African Trypanosomiasis.

i.v. – intra-venous; i.m – intra-muscular (Kennedy, 2004)

## **1.2 Sphingolipids**

### **1.2.1 Introduction**

Sphingolipids (SLs) were named because of their enigmatic, 'sphinx-like' properties by J. L. W. Thudichum who discovered this membrane component in 1884 (Futerman and Hannun, 2004). These lipid species can be found in all eukaryotic cells (Hannun et al., 2001) and have been demonstrated to be pivotal in signal transduction and cell membrane architecture (Futerman and Riezman, 2005).

Animals, including mammals, produce the phosphosphingolipid (PSL) sphingomyelin (SM) via the enzyme SM synthase (Huitema et al., 2004). However, plants, yeast and some protozoa produce inositol phosphorylceramide (IPC) via IPC synthase (Becker and Lester., 1980). Unlike the synthesis of sterols and glycerolipids, SL biosynthesis begins in the endoplasmic reticulum (ER) with the production of the unmodified sphingolipid ceramide, and then proceeds in the Golgi apparatus with synthesis of complex species (Futerman and Riezman, 2005).

In humans, SLs are associated with diseases including diabetes (Summers, 2005), some cancers (Modrak et al., 2006) and infection by microorganisms (Cowart and Obeid, 2006) in various ways. This evident importance has led to increasing research efforts focusing on the understanding of SL metabolism and function.

### 1.2.2 Structure of sphingolipids

The basic structure of SL consists of a long-chain sphingoid base connected to a fatty acid at the second carbon (C-2) by an amide bond (figure 1.7, Heung et al., 2006, Futerman and Hannun, 2004).

Complex PSLs are amphipathic molecules that contain both hydrophobic and hydrophilic regions (Futerman and Hannun, 2004). These hydrophobic (polar) groups are attached at the C-1 position of the sphingoid base through an ester bond (figure 1.7, Heung et al., 2006, Futerman and Hannun, 2004). Alternatively, the sphingoid base can be connected to one or more saccharide groups including glucose, galactose and N-acetylgalactosamine via a glycosidic bond to form complex, neutral glycosphingolipids (GSLs; figure 1.7, Masserini and Ravasi, 2001, Futerman and Hannun, 2004).

In yeast, sphingoid bases such as phytosphingosines and sphinganine carry 18-20 carbon atoms (C18-20; Merril, 2002, Rao et al., 2007). In mammals, the major SLs contain a C18 sphingoid base (Shayman, 2000) compared to C14 in insects (Lester and Dickson, 1993) and C16 in nematodes (Holthuis et al, 2001). More than 20 kinds of fatty acids can be found attached to the sphingoid base leading to a wide variety of SL species (Futerman and Riezman, 2005, Futerman and Hannun, 2004).

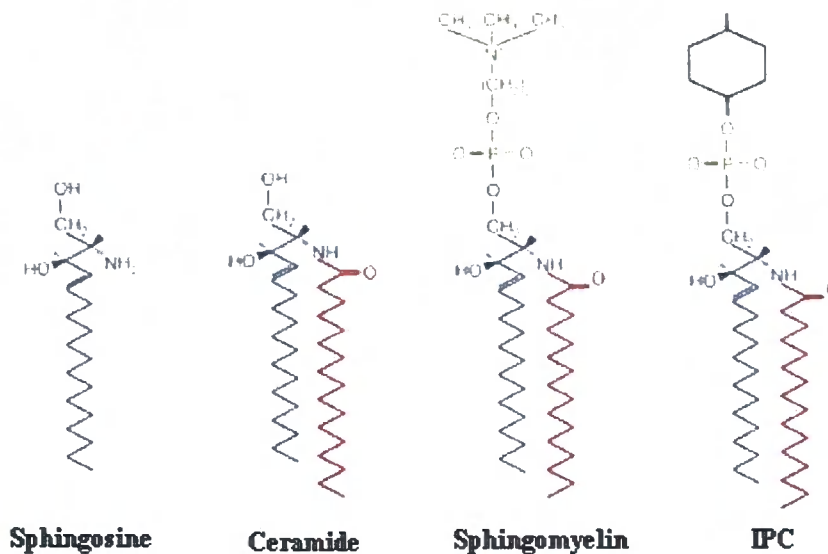


Figure 1.7  
Structure of sphingolipids (Futerman and Hannun, 2004).

### 1.2.3 Function

SLs play an important role in eukaryotic cells participating in the endo- and exocytosis signaling and cell-cell interactions (Rao et al, 2007). SLs in animals together with sterols, form lipid raft microdomains which mediate the recruitment of certain lipidated proteins to form receptors which mediate signal transduction, cell adhesion, migration and proliferation (Holthuis et al., 2001). However they also play a role in the resistance to chemotherapy (e.g. of anti-cancer drugs) and act as portals for microorganisms such as bacteria and viruses to enter the mammalian cell (Heung et al., 2006; Futerman and Hannun, 2004). In other eukaryotes, notably the fungi, SLs mediate the heat stress response, signal transduction and the pathogenesis of certain medically important species (Heung et al, 2006).

Furthermore, ceramide, the simplest SL in all eukaryotic cells, is found to promote cell apoptosis and inhibit cell proliferation (Futerman and Hannun, 2004).

#### 1.2.4 Biosynthetic pathway

##### 1. Ceramide synthesis (figure 1.8)

The first, rate-limiting step of sphingolipid synthesis is the reaction of L-serine and palmitoyl Co-A to form 3-ketosphingosine which is catalyzed by serine palmitoyl-transferase (SPT), the gene products of LCB1 and LCB2 in yeast (Nagiec et al., 1994). Subsequently, 3-ketosphingosine is reduced to form sphinganine by 3-ketosphingosine reductase which is encoded by TSC10 (Cowart and Obeid, 2007, Beeler et al., 1998).

In animal cells, sphinganine is N-acylated to form dihydroceramide and trans-double bonds are inserted at the 4, 5 position of sphinganine group to form ceramide (Merrill et al., 1997). In yeast, sphinganine is hydroxylated to form phytosphingosine (Grilley et al., 1998). (Phyto)ceramide is subsequently incorporated into many SLs such as ceramide-1-phosphate, SM and GSLs.

##### 2. Sphingomyelin synthase (figure 1.19)

The formation of ceramide occurs in the ER (Rao and Acharya, 2007) and it's transported from ER to Golgi apparatus by a specific ceramide transport molecule (CERT; Hanada et al., 2003) in animal cells. After that, Phosphocholine is transferred from phosphatidylcholine (PC) to ceramide to form SM in Golgi apparatus and at the plasma membrane (Futerman and Hannun, 2004). SM can also be degraded into ceramide by sphingomyelinase (SMase), a process which regulates cell growth and apoptosis (Hannun and Luberto, 2004).

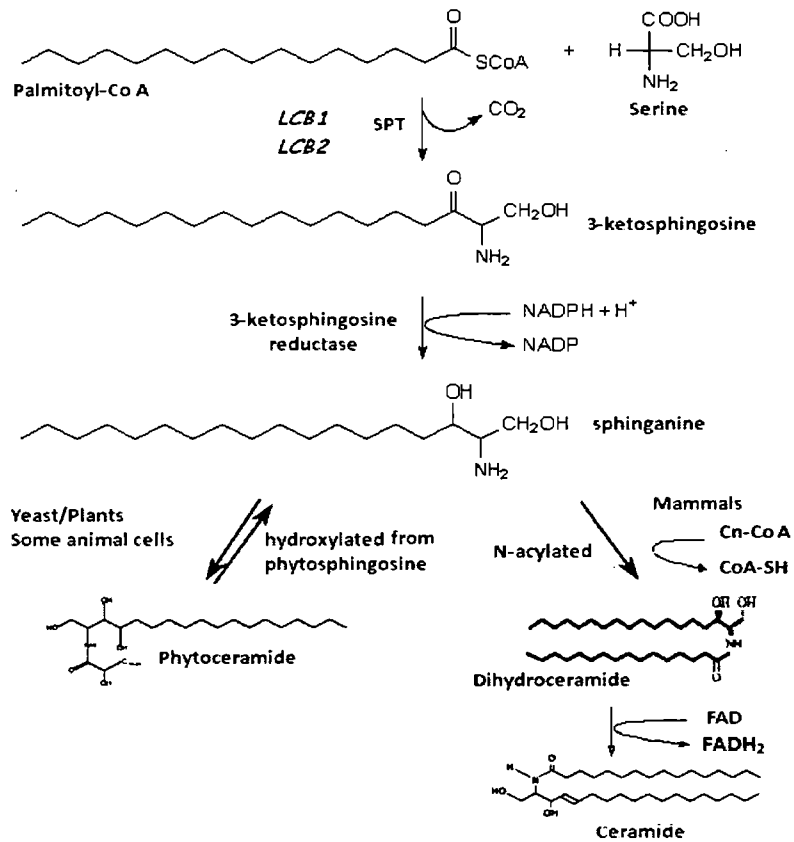


Figure 1.8  
Pathway of ceramide synthase (Holthuis et al., 2001). The formation of creamide is in mammals and phytoceramide in yeast or plants.

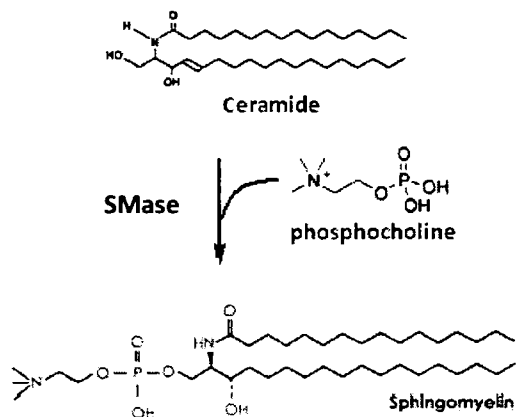


Figure 1.9  
Pathway of SM synthesis (Futerman and Hannun, 2004). Phosphocholine is transferred from phosphatidylcholine (PC).

### 3. Inositol ceramide synthesis (figure 1.10)

In yeast, plants and some protozoa, the phosphoinositol head-group from phosphatidylinositol (PI) is transferred to phytoceramide to form inositol phosphorylceramide (IPC), a process catalyzed by IPC synthase (*AUR1p* in yeast). Like mammals, the complex sphingolipid, IPC, is synthesized in the Golgi apparatus (Levine et al., 2000; Denny et al. 2006). In yeast, IPC is subsequently mannosylated by the gene products of *CSG1*, *CSG2* and *CSH1* to form mannoseinositol phosphor-ceramide (MIPC, Beeler et al., 1997). Subsequently, another inositol phosphate is added to MIPC to form manose inositol (P)<sub>2</sub> ceramide (M(IP)<sub>2</sub>C), a process regulated by the enzyme encoded by *Ipt1* (Dickson et al., 1997).

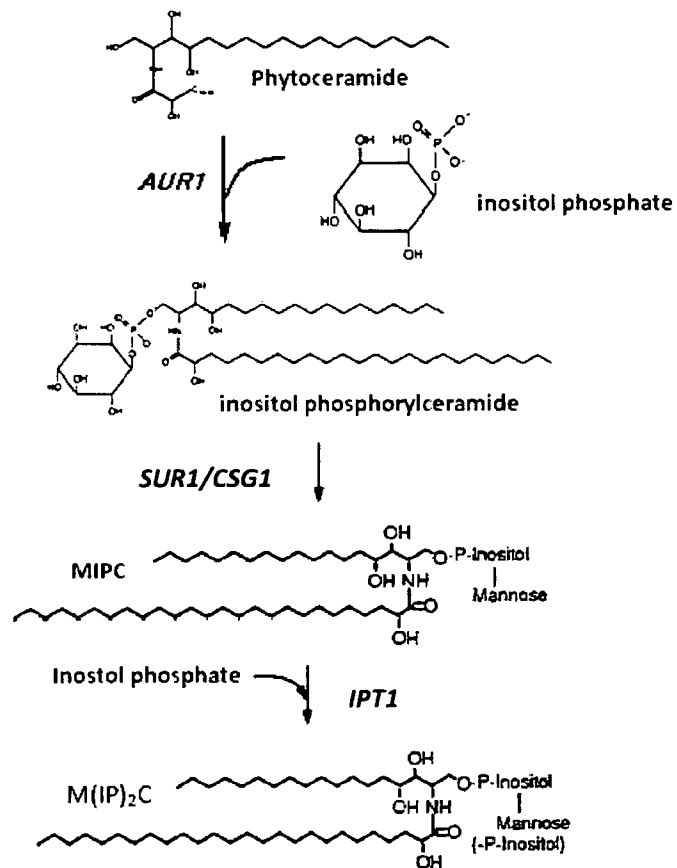


Figure 1.10

Pathway of IPC synthesis (Beeler et al., 1997). Genes are shown in capital italics.

### 1.2.5 Sphingolipid biosynthesis as a drug target

So far, 11 enzymes have been found to be involved in SLs metabolism (Futerman and Riezman, 2005). SPT, the first enzyme in the pathway, belongs to the pyridoxal phosphate-dependent  $\alpha$ -oxoamine synthase family. Drugs are known to specifically inhibit the function of SPT: sphingofungin, lipoxamycin and myriocin (figure 1.11, Delgado et al., 2006). In addition, ceramide synthesis can be inhibited by fungi metabolites such as fumonisin produced by *Fusarium verticilloides* (figure 1.12, Desai et al., 2002), the fumonisin-like AAL-toxin produced by *Alternaria alternate* var. *lycopersici* (figure 1.12, Winter et al., 1996) and australifungins from *Aporormiella australis*. (Mandala et al., 1995)

IPC synthase activity is evident only in yeast, plants and protozoa rather than animal cells. The inhibition of IPC synthase has become an important target in the fight against these pathogens (Nagiec et al., 1997). Inhibition by aureobasidin A (produced by *Aureobasidium pullulans*, Zhong et al., 2000) has been demonstrated in many yeast and fungi including *S. cerevisiae*, and the pathogenic *Candida* and *Aspergillus* species (Zhong et al., 2000). Kinetoplastid parasites including *Leishmania major* (Heung et al., 2006) and *Trypanosoma cruzi* (Figueiredo et al., 2005) also have IPC synthase activity and reports indicate that aureobasidin A successfully inhibits the IPC synthase from these protozoan parasites (Denny et al., 2006, Figueiredo et al., 2005). Therefore, IPC synthase represents a new drug target for the pathogenic protozoa.



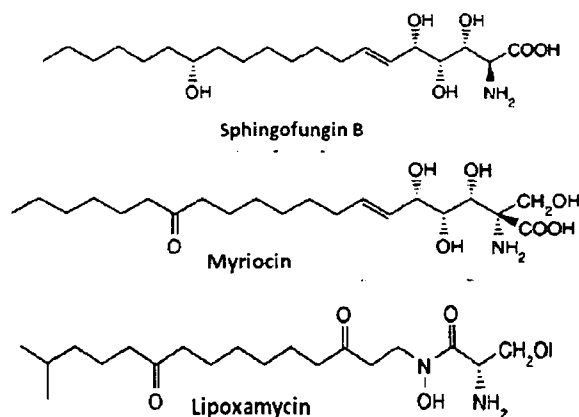


Figure 1.11  
Structure of drugs used to inhibit SPT (Delgado et al., 2006). These compounds react with SPT to interfere the the catalytic reaction.

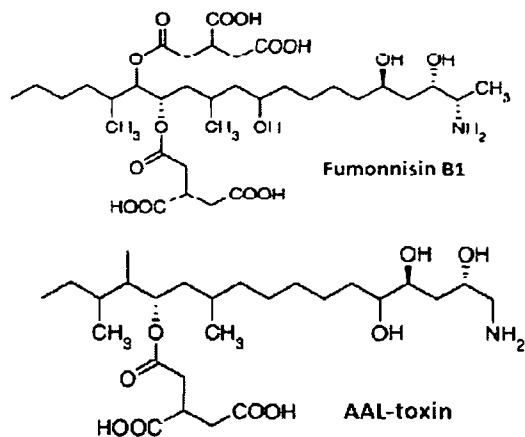


Figure 1.12  
Structure of drugs used to inhibit ceramide synthase (Delgado et al., 2006). Both inhibitors bind to the dicarboxylic acid side chains on the sphingoid base substrate.

### **1.3 The *Trypanosoma brucei* sphingolipid synthase**

The identification of kinetoplastid IPC synthase (Denny *et al.*, 2006) has made possible: i) its validation as an anti-protozoal target; and ii) the establishment of the specific efficacy of known fungal IPC synthase inhibitors. The *T. brucei* database contains four closely related orthologues of the characterised *Leishmania* IPC synthase (Denny *et al.*, 2006). Previously, mass spectrometry of lipid fractions has shown that whilst the major phosphosphingolipid in pathogenic bloodstream form parasites is SM (Patniak *et al.*, 1993; Richmond *et al.*, 2007a; Richmond *et al.*, 2007b); insect stage, procyclic *T. brucei* also contain, like *Leishmania*, IPC (Güther *et al.*, 2006).

This report describes the characterization of the *T. brucei*, sphingolipid synthase (*TbSLS*) which demonstrates itself to be an IPC synthase, but with the further ability to mediate the production of 2 other unknown sphingolipids (X – co-migrating with SM; and Y). The IPC synthase activity of *TbSLS* is acutely sensitive to the fungal inhibitor aureobasidin A and bloodstream form *T. brucei* are rapidly killed at sub-micromolar concentrations of this drug. Down-regulation of *TbSLS*, using inhibition RNA (RNAi), in bloodstream form parasites demonstrated that the enzyme activity is essential for growth thus validating it as a target for the development of new therapies.

## 1.4 What is RNAi?

The interference RNA (RNAi) response was first identified in plants (Napoli et al., 1990) and is a natural biological protective response to viral dsRNA (Sledz and Williams, 2005). However, since Fire *et al* (1998) injected dsRNA into *Caenorhabditis elegans* and found that gene expression could be specifically inhibited the technology of RNAi, in which the Dicer and RISC complexes play an important role, has developed very quickly as tool for studies of gene function (Geley and Muller, 2004). RNAi now is found to function in many eukaryotic organisms such as *Drosophila*, hydra, zebrafish, mammals and *Trypanosoma brucei* (table 1.2, Brantl, 2002).

kingdom	species	Stage tested	Delivery method
Protozoans	<i>Trypanosoma brucei</i>	Procyclic and bloodstream forms	Transfection
	<i>Toxoplasma gondii</i>	Mature form in fibroblast	Transfection
Invertebrates	<i>Drosophila melanogaster</i>	Cell lines, adult and embryo	Injection for adult and embryo stage, soaking and transfection for cell lines
	Hydra	Adult	Delivered by micropipette
Vertebrates	Zebra fish	Embryo	Microinjection
	Mice	Prenatal, embryonic stages and adult	Injection
	Human	Human cell lines	Transfection
Plants	Onocots/dicots	Plant	Particle bombardment with siRNA/transgenics
Fungi	<i>Neurospora crassa</i>	Filamentous fungi	Transfection
	<i>S. pombe</i>	Filamentous fungi	Transgene
Algae	<i>Chlamydomonas reinhardtii</i>		Transfection

Table 1.2

Eukaryotic organisms exhibiting RNA-related phenomena. (Brantl, 2002)

# **Chapter 2:**

## **Materials and Methods**

## **Chapter2: Materials and methods**

### **2.1 Materials**

#### **2.1.1 Chemicals**

Tris/HCl, glucose, ammonium sulphate, galactose, KCl, lithium acetate, raffinose, tryptophan,  $\beta$ -mercaptoethanol, NaCl, chloroform (A.C.S), methanol (A.C.S) and acid-washed glass beads (425-600 $\mu$ m, 30-40 U.S. sieve) were from Sigma Aldrich.

Yeast nitrogen base and BODIPY FL-ceramide were from Invitrogen

HMI-9 powder, penicillin/streptomycin, foetal bovine serum and L-glutamine were from GIBCO BRL.

G418, agarose, tryptone and yeast extract were from Melford.

Amino acids drop-out supplement –HIS-URA-TRP was from Clontech.

Complete EDTA-free protease inhibitor cocktail tablets and HPTLC plates were from Merck.

### 2.1.2 Buffers

<b>Solution</b>	<b>Final conc.</b>	<b>Volume</b>	<b>Stock solution</b>
<b>STE</b>	25mM	1.25ml	Tris/HCL(1M)
<i>(50ml)</i>	250mM	12.5ml	Sucrose(1M)
	1mM	100µl	EDTA(0.5M)
		1 tab	Complete protease inhibitor cocktail
		Add to 50ml	dH <sub>2</sub> O
<b>Storage buffer</b>	50mM	2.5ml	Tris/HCL
<i>(50ml)</i>	15%w/v	6.25ml	Glycerol(80%w/v)
	5mM	0.25ml	MgCl <sub>2</sub> (1M)
		1 tab	Complete protease inhibitor cocktail
		Add to 50ml	dH <sub>2</sub> O
<b>Tris/EDTA/BSA</b>	250mM	12.5ml	Tris/HCL(1M)
<b>buffer</b>	25mM	2.5ml	EDTA(0.5M)
<i>(50ml)</i>	15mg/ml	750mg	Fatty-acid-free BSA
		Add to 50ml	dH <sub>2</sub> O

### 2.1.3 Media

<b>Media</b>	<b>Weight</b>	<b>Compositions</b>
<b>SD-HIS-URA</b>	20gm	Glucose
<i>(1L)</i>	1.7g	Yeast nitrogen base
	5g	Ammonium sulphate
	0.7g	Amino acid drop-out supplement –HIS-URA-TRP
	1ml	Tryptophan (4mg/ml)
	20g	Agar (added for solid media preparation)
	Add to 1000ml	dH <sub>2</sub> O
<hr/>		
<b>YPGR</b> <i>(1L)</i>		
<i>Sol I</i>	10g	Yeast extract
	20g	Peptone
	800ml	Galactose
<i>Sol II</i>	40g	Raffinose
	20g	dH <sub>2</sub> O
	200ml	
<hr/>		
<b>SGR</b> <i>(1L)</i>		
<i>Sol I</i>	40g	Galactose
	20g	Raffinose
	900ml	dH <sub>2</sub> O
<i>Sol II</i>	3.5g	Amino acid drop-out supplement –HIS-URA-TRP
	100ml	dH <sub>2</sub> O

<b>Media</b>		<b>Weight</b>	<b>Compositions</b>
<b>SOC</b>	<i>(1L)</i>	20g	Tryptone
		5g	Yeast extract
		0.584g	NaCl
		0.186g	KCl
		2.003g	MgCl <sub>2</sub>
		2.465g	MgSO <sub>4</sub>
		3.6g	Glucose
<b>HMI-9</b>	<i>(1L)</i>	10.14g	HMI-9 powder
		3.024g	NaHCO <sub>3</sub>
		14.3ul	2M β-mercaptoethanol
		10ml	Penicillin/streptomycin
		10ml	L-Glutamine
		100ml	Foetal bovine serum
		to 1L	dH <sub>2</sub> O
<b>LB</b>	<i>(1L)</i>	10g	Tryptone
		5g	Yeast extract
		10g	NaCl
		Add to 1000ml	dH <sub>2</sub> O



### 2.1.4 Antibiotics

<b>Antibiotics</b>	<b>Stock conc.</b>	<b>Store temperature</b>	<b>Final conc.</b>
Ampicillin	50mg/ml	-20°C	50µg/ml
Doxycyclin	1mg/ml	-20°C	1µg/ml
Phleomycin	5mg/ml	-20°C	2.5µg/ml
G418	5mg/ml	-20°C	2µg/ml

### 2.1.5 Strains and vectors

<b>Strains/ Vectors</b>	<b>Work</b>
DH5a	DNA cloning
<i>S. cerevisiae</i> HIS-GAL AUR1 <i>TbSLS1000</i>	Mutant yeast complemented by <i>T. brucei</i> SLS
Single Marker Bloodstream form (SMB, T7RNAP::TETR::NEO)	Aureobasidin A efficacy and RNAi induction
pRS426	Expression of of <i>TbSLS</i> in yeast
p2T7	RNAi

## 2.2 Bioinformatic and *ex vivo* analyses

### 2.2.1 *T. brucei* sequence analysis

There are four *T. brucei* sphingolipid synthase (*TbSLS*) sequence orthologues evident in the *T. brucei* database ([www.geneDB.org](http://www.geneDB.org)). Sequence alignments were performed using ClustalW ([www.clustal.org](http://www.clustal.org)). *Tb09.211.1000* was analysed here.

### 2.2.2 Metabolic labeling

*S. cerevisiae* HIS-GAL AUR1 *TbSLS1000* and controls were grown to exponential phase (OD<sub>600</sub> 0.5-0.7) and 10 units (OD<sub>600</sub>=1=1 unit) of cells pelleted, resuspended in 250µl of SD-HIS-URA media and transferred into LoBind Eppendorf tubes. 5µl of 5mM BSA-conjugated FL-ceramide and/or 50µCi C<sup>14</sup>Choline (GE Healthcare) was added and the cells were incubated with shaking at 30°C for 2 hours. The cells were washed with 1ml of dH<sub>2</sub>O and 400µl of chloroform:methanol (C:M; 1:1 v/v) added to the pellet before disruption with 0.1-0.2g of glass beads in a vortex mixer at full speed for 10 seconds. 400µl of C:M:dH<sub>2</sub>O (C:M:W; 10:10:3 v/v/v) was added to the supernatant. The organic, lipid fraction was isolated by biphasic separation in a Folch wash, before being dried at 30°C in a rotary concentrator (Eppendorf 5301). The samples were then resuspended in 20ul of C:M:W, 10:10:3 and fractionated by HPTLC with C:M:0.25%KCl, 55:45:10 as the solvent phase. For 2-dimensional chromatography lipid extracts were fractionated in the first dimension with C:M:4.2M NH<sub>4</sub>OH (9:7:3) as the solvent phase and with C:M:Acetic Acid (45:30:7). Subsequently, the plates were scanned using a Fuji3000 fluorescent scanner and analyzed using AIDA Analyzer software (imaGenes). Radiolabelled samples were detected after spraying with EN<sup>3</sup>HANCE™ (DuPont) by exposure to BioMax (Kodak) film. Bloodstream form *T. brucei* (SMB) were similarly labeled but with 10<sup>7</sup> in

serum-free HMI-9 at 37°C, after a 1 hour incubation in the same media. Labeled mammalian, CHO cell controls were provided by Dr Steven Pratt.

### 2.2.3 Mass spectrometry analyses of *S. cerevisiae* HIS-GAL AUR1

#### *TbSLS1000*

$2 \times 10^9$  freeze-dried yeast cells were extracted twice with 250ml of C:M:W, 4:8:3 in a sonicating water bath for 2 hours and centrifuged. The supernatants were adjusted to C:M:V, 4:8:5.6, vortexed and centrifuged. The lower phase was loaded into nanospray tips (Micromass type F) and analysed by electrospray mass spectrometry (ES-MS and ES-MS-MS) using a Micromass Quattro Ultima triple quadrupole mass spectrometer in positive- and negative-ion modes. Capillary and cone voltages were 0.9 kV and 30 V (negative ion) and 1.2 kV and 50 V (positive ion), respectively. Argon was used as the collision gas ( $3.0 \times 10^{-3}$  Torr) to collect tandem spectra (ES-MSMS). Collision energies for parent ion scanning were: 60 V, negative-ion parents of  $m/z$  241 for inositol-phospholipids; 37 V, positive-ion parents of  $m/z$  184 for phosphate dylcholines. Collision energies for neutral-loss scanning were: 20 V, for neutral-loss  $m/z$  141 scanning for phosphatidylethanolamines; 25 V, for neutral-loss  $m/z$  181 for phosphatidylserines. Negative-ion daughter ion spectra were collected with a collision energy of 60 V.

#### 2.2.4 Agar diffusion assay

*Saccharomyces cerevisiae* HIS-GAL AUR1 complemented with *TbSLS1000* (provided by Dr Paul Denny) were grown in SD-HIS-URA media overnight at 30°C. Cells were diluted into 80ml of SD-HIS-URA media so that OD<sub>600</sub> was approximately 0.2, and incubated until the OD<sub>600</sub> was 0.5-0.7 (exponential phase).  $2.4 \times 10^7$  cells (OD<sub>600</sub>=1=10<sup>7</sup>/ml cells) were pelleted in a benchtop centrifuge at 3000rpm for 5 minutes. SD-HIS-URA solution I with 2% agarose and solution II were warmed to 45°C. 13.5ml solution I with 2% agarose and 1.5ml of SD-HIS-URA solution II were then mixed with the cell pellet and the mixture poured into square Petri dishes. When set inhibitors at appropriate concentrations in DMSO were applied and the plates then incubated at 30°C for three to four days.

### 2.3 *In vitro* enzyme assay

#### 2.3.1 Preparation of microsomes

5L of cells, grown to exponential phase as above (2.3.1), were pelleted at 6000rpm, 15 minutes, 4°C and washed twice with ice-cold PBS. An equal volume of chilled glass beads and STE buffer were added to the pellet and the cells disrupted by vortex mixing. The mixture was centrifuged at 5000rpm, 10 minutes, 4°C and the supernatant transferred to a 50ml tube. The pellet was re-extracted as before and then supernatants combined and a microsomal fraction isolated by differential centrifugation: 147000rpm, 35 minutes, 4°C in an SW60Ti rotor to remove the large granular fraction, followed by 35000rpm, 95 minutes, 4°C to pellet the small granular fraction containing microsomal membranes. The supernatant was removed and the microsomal membrane resuspended in 100µl of storage buffer. The protein concentration was established using the Lowry method (Lowry et al, 1951), adjusted to

10mg/ml and the membranes were stored at -80°C until use.

### 2.3.2 Preparation of washed microsomes

Equal volumes of the microsomal membrane preparation and a 2.5% CHAPS solution in STE were mixed together and incubated on the ice for 60 minutes. Subsequently the mixture was centrifuged at 43000rpm for 100 minutes at 4°C in a SW60Ti rotor, the pellet resuspended in 50ul of storage buffer and stored at -80°C until required.

### 2.3.3 *In vitro* TbSLS1000 assay

5µl of 10mM PI, 4µl of 13mM PE or 4µl of 13mM PC were added into LoBind tubes and air dried. 20µl of Tris/EDTA/BSA buffer was added and the samples sonicated in a water bath for 2 minutes. 10µg of unwashed (crude) or equivalent washed microsomes were added into each tube and the volume adjusted to 48µl with dH<sub>2</sub>O. 2µl of 5mM BSA conjugated BODIPY FL-ceramide were added in each tube and the reactions incubated at 30°C for 1 hour. 150µl of C:M:W, 10:10:3 was added and, after centrifugation, the lower organic phase was removed to another LoBind tube. The samples were subsequently analysed by HPTLC as above.

### 2.3.4 Optimum temperature for *TbSLS* activity *in vitro*

20µl of Tris/EDTA/BSA buffer, 1µl of 10mg/ml unwashed (crude) microsomes were added to LoBind Eppendorf tubes and dH<sub>2</sub>O were added to 48µl. These tubes were preincubated at 4°C, 20°C, 30°C, 33°C, 37°C and 40°C for 30 minutes, then 2µl of 5mM BSA conjugated BODIPY FL-ceramide added and the reaction incubated at the appropriate temperature for another 1 hour. The samples were processed and analysed by HPTLC as above.

### 2.3.5 Efficacy of aureobasidin A against *Tb*SLS activity *in vitro*

20 µl of Tris/EDTA/BSA buffer were added into Lobind Eppendork tubes. 5µl of different concentrations of aureobasidin A were added together with 1µl of 10gm/ml unwashed (crude) microsomes and dH<sub>2</sub>O to 48µl. The samples were preincubated at 30°C for 30 minutes, then 2µl of 5mM FL-ceramide were added in each tube and the reaction incubated at the 30°C for another 1 hour. The samples were processed and analysed by HPTLC as above.

## 2.4 Cellular analyses

### 2.4.1 Parasite culture

The bloodstream form *T. brucei* strain Lister 427 engineered variant, Single Marker Bloodstream form (SMB, T7RNAP::TETR::NEO; Wirtz et al., 1999), was maintained *in vitro* at 37°C with 5% CO<sub>2</sub> in HMI-9 medium supplemented with 10% FCS and 2.5µg/ml G418.

### 2.4.2 RNAi

Primers (SigmaGensys) were designed to amplify 2 fragments (approximately 180bps) completely conserved between the four copies of *Tb*SLS.

Primers	Sequences
5' <i>Tb</i> SLS RNAi1 XbaI	CATAGATCTAGAAAAGTACCTTCTTCACCG
3' <i>Tb</i> SLS RNAi1 XbaI	CATAGATCTAGAAGCAGGAGTGCGTATGATG
5' <i>Tb</i> SLS RNAi2 XbaI	CATAGATCTAGAGGTTCCATACACTGTG
3' <i>Tb</i> SLS RNAi2 XbaI	CATAGATCTAGACGAGAGGCAACGATGC

These primers were used to amplify the fragments from genomic *T. brucei* DNA (1µg) using FidelityTaq (USB) according to manufacturer's protocols.

Engineered to contain 5' and 3' XbaI sites these 2 fragments were subsequently digested using XbaI (Promega) and ligated using T4 ligase (Promega) into the p2T7 RNAi vector before selection in DH5a *Escherichia coli*.

A *Tb*LCB2 (SPT subunit 2) fragment cloned into p2T7 was provided by Dr P. Denny.

p2T7 constructs were amplified in *E.coli* and isolated by Midi Prep (Qiagen) before being linearized by NotI digestion, at least 10µg of DNA was used for each transfection.  $2.5 \times 10^7$  cells were grown for each transfection and pelleted at 1000rpm, 10minutes at room temperature in a benchtop centrifuge. Cells were washed with 25ml of cytomix, then resuspended in the same at  $6 \times 10^7$ /ml). 10µg of linear plasmid in 10µg was mixed with 450µl of cells in an Eppendorf tubes, transferred into 2mm gap electroporation cuvettes (Sigma) and pulsed at 1.4kV, 2.5µF in a BioRad Gene Pulser II. After electroporation, cells were transferred into 36ml of HMI-9 media and incubated at 37°C, 6 hours before adding 7.2µl of 5mg/ml G418 and 18µl of 5mg/ml phleomycin. Parasites were transferred into 24 well plates with each well containing 1ml of cells. Plates were incubated at 37°C for approximately six days and the selected transfectants sub-cultured.

Two flasks of RNAi transfected SMB cells were grown in HMI-9 with 2.5µ/ml phleomycin and 2.5µg/ml G418 with an initial cell density of  $1 \times 10^5$ /ml. 1µg/ml of doxycyclin was added into one flask, the other acted as a control. Parasites were incubated at 37°C and cell counts and observations made after 24, 48 and 72 hours. Cells were sub-cultured to  $1 \times 10^5$ /ml when they reached or exceeded  $2 \times 10^6$ /ml.

RNA was extracted from approximately  $10^7$  parasites using the RNeasy kit (Promega) as per the manufacturer's protocol except that that cells were disrupted using glass beads in 600 $\mu$ l of RTL buffer prior to extraction. Isolated RNA was treated with DNaseI (Promega) at 37°C, 30 minutes before 3 $\mu$ g was used to make cDNA using the 3' primers above and SuperScript II (Promega) according to the manufacturer's protocols. Subsequently, the primers below were used to amplify cDNA fragments using PCR Master Mix (Promega) and the products then analyzed by agarose gel electrophoresis.

#### 2.4.3 Aureobasidin A efficacy against *T. brucei*

*T. brucei* SMB were cultured in the presence of various concentrations of aureobasidin

A. Growth was analyzed at 24 hour intervals as below.

<b>Primers</b>	<b>Sequences</b>
5' <i>Tb</i> SLS	AAACTGTACCTTCACCG
3' <i>Tb</i> SLS	AGCAGGAGTGCGTATGATG
5' <i>Tb</i> BTUB	GGAGCGCATCAATGTGTAC
3' <i>Tb</i> BTUB	CAGGCAGCAGGTGACGCCG
5' <i>Tb</i> LCB2	ATGCCTACTTACGTGGAGGC
3' <i>Tb</i> LCB2	CGTCGAAACCCGCTCCATCAC



# **Chapter 3:**

## **Results**

## Chapter 3: Results

### 3.1 General information

African sleeping sickness which is listed as a tropical disease research (TDR) category 1 disease is emerging as a serious problem in parts of sub-Saharan Africa and new drugs are urgently needed ([www.who.int/tdr/](http://www.who.int/tdr/)). According to the 2004 WHO report, 48000 people lost their lives to this disease in that year. Compared to humans and other mammals, pathogenic protozoa and fungi have differences in their sphingolipid biosynthetic pathways. This difference offers target sites for novel inhibitor molecules and the opportunity for developing new drugs (Heung et al., 2006).

This report describes experimental work comprising 3 parts.

1. Functional identification and characterization of the *Trypanosoma brucei* sphingolipid synthase (*TbSLS*).
2. The inhibition of *T. brucei* sphingolipid synthase using a known anti-fungal drug aureobasidin A.
3. Functional analysis using RNA interference (RNAi).

## 3.2 Identification and characterisation of the *T. brucei* sphingolipid synthase

### 3.2.1 *T. brucei* sphingolipid synthase (*TbSLS*) complements an AUR1 mutant yeast

Four gene sequences (*Tb09211.1000*, *Tb09.211.1010*, *Tb09.211.1020* and *Tb09.211.1030*) were previously identified in the genome database ([www.genedb.org](http://www.genedb.org)) as sequence orthologues of the inositol phosphorylceramide synthase (*LmIPCS*) from the related kinetoplastid *Leishmania* parasite (Denny et al., 2006). Further analysis of the genomic sequence identified the complete open reading frames (ORFs) found on chromosome 9 which are predicted to encode 4 transmembrane proteins with more than 70% identity. Most variation occurs at the carboxy-termini, a region predicted to lie on the cytosolic side of the membrane away from the active site at the Golgi lumen (Denny et al., 2006, figure 3.1). Like *LmIPCS*, *TbSLS* possesses conserved sphingomyelin (SM) synthase domains: D1, D3 and D4 (Huitema et al., 2004). D3 (C-G-D-X<sub>3</sub>-S-G-H-T) and D4 (H-Y-T-X-D-V-X<sub>3</sub>-Y-X<sub>6</sub>-F-X<sub>2</sub>-Y-H) are similar to the C2 and C3 motifs in Lipid-Phosphate Phosphatase (LPP, Neuwald, 1997) and domains 3 and 4 of the fungal AUR1p (Heidler and Radding, 2000). D3 and D4 contain the histidine and aspartate residues (underlined) that form the catalytic triad which mediates nucleophilic attack on lipid phosphate ester bonds (Huitema et al., 2004; Neuwald, 1997). Gene mutation at this triad inhibits both the fungal and *Leishmania* IPC synthase (Levine et al., 2000; Mina and Denny, personal communication). One of the predicted *T. brucei* sphingolipid synthase isoforms, the longest (*Tb09.211.1000*, *TbSLS1000*), was analysed in this study.

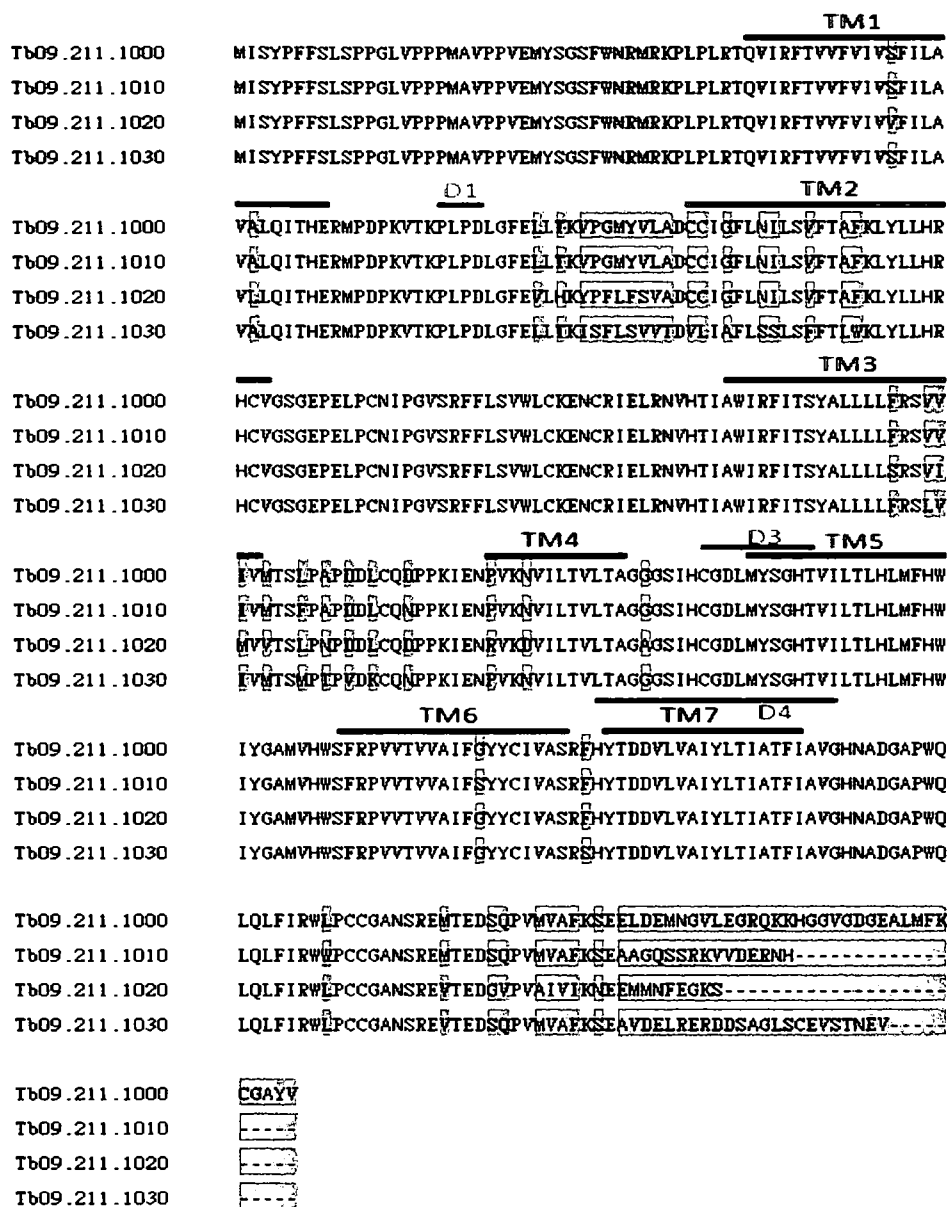


Figure 3.1:  
 Predicted protein sequences of the 4 *T. brucei* ORFs aligned using ClustalW 1.81 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The blue regions indicate the non-identical regions. TM – predicted transmembrane domains (Denny et al., 2006); D1, D3 and D4 – conserved SM synthase domains.

The auxotrophic mutant *Saccharomyces cerevisiae* strain YPH499-HIS-GAL-AUR1 in which the essential AUR1 IPC synthase gene is under the control of a galactose inducible promoter is unable to grow in the absence of galactose. However, this can be rescued by the expression of *LmIPCS* (Denny et al., 2006). Similarly, *TbSLS1000* expression complemented the mutant cell indicating that *TbSLS1000* is a functional orthologue of the yeast AUR1 gene.

### 3.2.2 Functional identification of *T. brucei* SLS

To understand the function of *TbSLS1000*, the complemented yeast and pathogenic bloodstream (BSF) *T. brucei* were labeled with fluorescent BODIPY FL C<sub>5</sub>-ceramide (a substrate for sphingolipid synthases) complexed with BSA as described in the Materials and Methods. The lipids were extracted and analysed by the high performance thin layer chromatography (HPTLC). *S. cerevisiae* which produces IPC via the action of IPC synthase (Becker and Lester., 1980) and mammalian Chinese Hamster Ovary (CHO) cells which produce sphingomyelin (SM) via the action of SM synthase (Huitema et al., 2004) were similarly labeled and their products used as makers (figure3.2). The BSF parasites produce 2 labeled products: one that co-migrated with SM (X) and another (Y) which migrated beyond IPC. The complemented yeast produced 2 apparently similar species that co-migrated with the BSF products, as well as a faint band that co-migrated with IPC (figure 3.2). These data indicated that *TbSLS1000* functions primarily as a SM synthase.

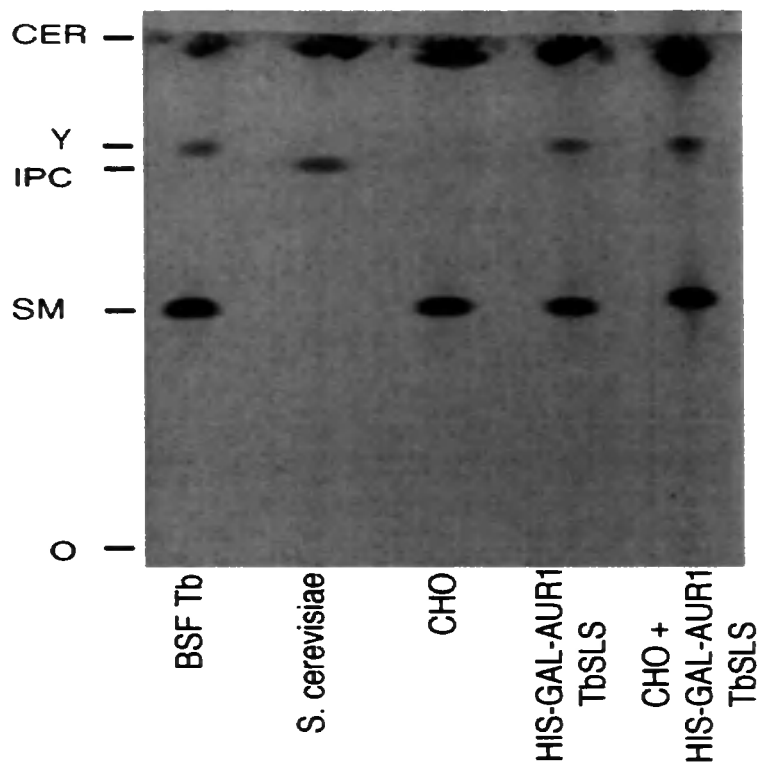


Figure 3.2:

Metabolic labelling. TbSLS1000, *S. cerevisiae* and mammalian cells (CHO) were labelled with BODIPY FL-ceramide. Samples were analysed by HPTLC.

CER – ceramide; Y – unknown;

IPC – inositol phosphorylceramide;

SM – sphingomyelin; O – origin;

BSF Tb – bloodstream form *T. brucei*; CHO – Chinese Hamster Ovary cells;

HIS-GAL-AUR1 TbSLS – auxotrophic mutant yeast complemented with TbSLS1000

### 3.2.3 2-dimensional HPTLC analyses

In order to further characterize the labeled sphingolipids extracted from the *TbSLS1000* complemented yeast and the *T. brucei* bloodstream form parasites, 2D-HPTLC was used to further separate the different ceramide-labeled species produced. 2 labeled lipid fractions were loaded at the same origin on a HPTLC plate and separated in 2 dimensions using 2 different solvent systems.

As shown in figure 3.3, the lipids from the *TbSLS1000* complemented yeast and *T. brucei* BSF cells separated into three groups: apparent IPC (only produced by the complemented yeast), and unknowns X (shown to co-migrate with SM above), and Y. This indicated that the X and Y are produced in both *T. brucei* BSF cells and the complemented yeast; therefore, it seems that *TbSLS1000* (and, presumably, its isoforms) functions as the major sphingolipid synthase in the pathogenic form of *T. brucei*. IPC could only be detected in the complemented yeast (see above). Notably, this lipid has only been detected in insect stage, procyclic form *T. brucei* (Güther et al., 2006).

In one dimensional HPTLC, unknown X co-migrated with SM (see above). To further investigate the identity of X, labeled lipids from *T. brucei* BSF parasites and mammalian cells were analyzed by 2D-HPTLC. As seen in figure 3.4, SM and X could not be separated under these conditions. This supported the suggestion that X is SM and that *TbSLS1000* functions primarily as a SM synthase.

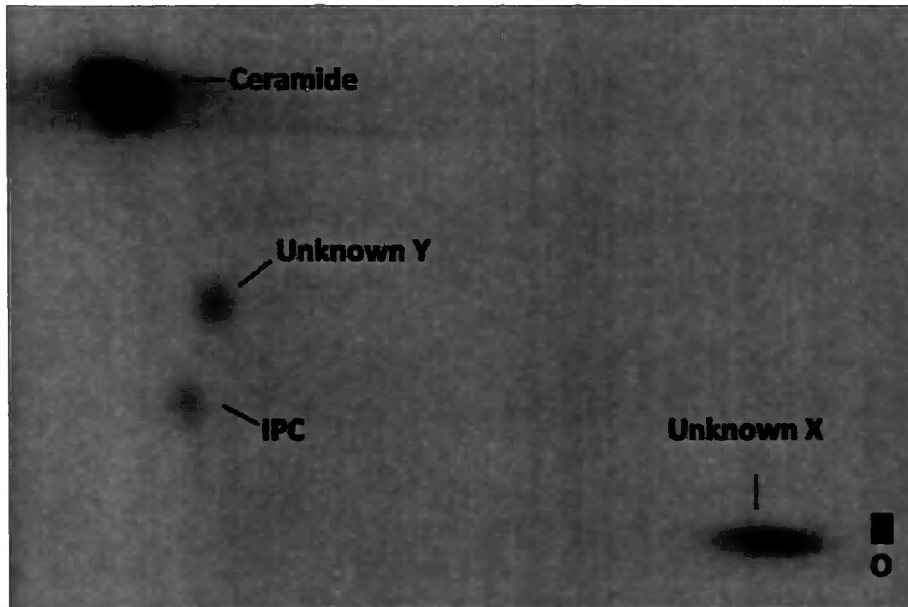


Figure 3.3:  
 2D HPTLC analysis. *T. brucei* SLS complemented yeast and *T. brucei* BSF parasites were labeled with BODIPY FL-ceramide and the lipids extracted and analysed by HPTLC. The samples were spotted at the same labeled origin and separated in 2 dimensions using 2 different solvent systems. IPC- Inositol phosphorylceramide

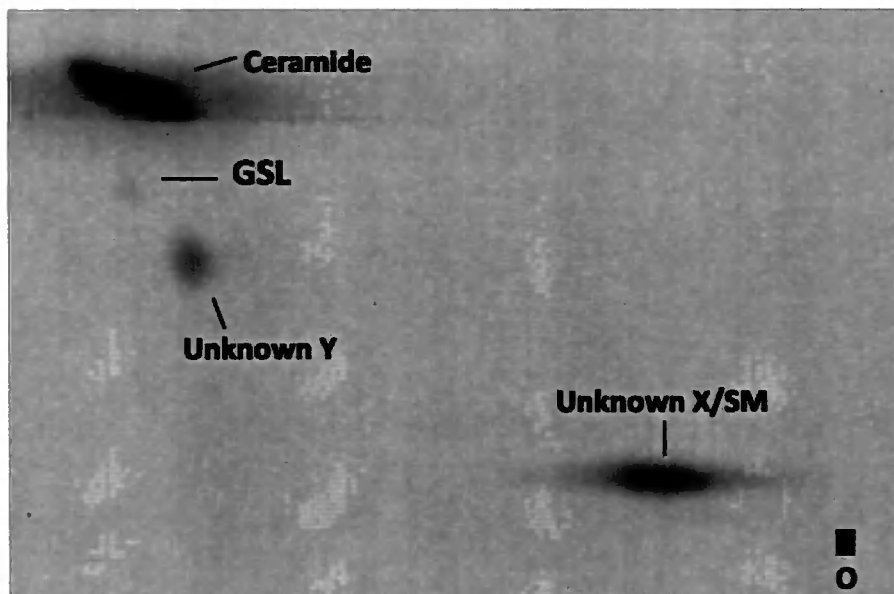


Figure 3.4:  
 2D HPTLC analysis. *T. brucei* BSF parasites and mammalian CHO cells were labeled with BODIPY FL-ceramide and the lipids extracted and analysed by HPTLC. The samples were spotted at the same labeled origin and separated in 2 dimensions using 2 different solvent systems. GSL – glycosphingolipid; SM- sphingomyelin.



In order to prove that *TbSLS1000* functions as SM synthase, complemented yeast were labeled with  $^{14}\text{C}$  choline and BODIPY FL-ceramide as described in Materials and Methods. The formation of SM via SM synthase requires phosphatidylcholine (PC) as the donor substrate (Huitema et al., 2004) and since  $^{14}\text{C}$  choline is incorporated into PC, radio isotope will also label any SM produced by the complemented yeast.

Figure 3.5 shows the result of this dual labeling when the lipids are separated by 2D-HPTLC. It is clear to see PC labeled on the plate; however, no  $^{14}\text{C}$  choline species were co-labeled with ceramide. This indicated that SM synthase activity is absent, X is not SM and that *TbSLS1000* complemented yeast do not produce SM.

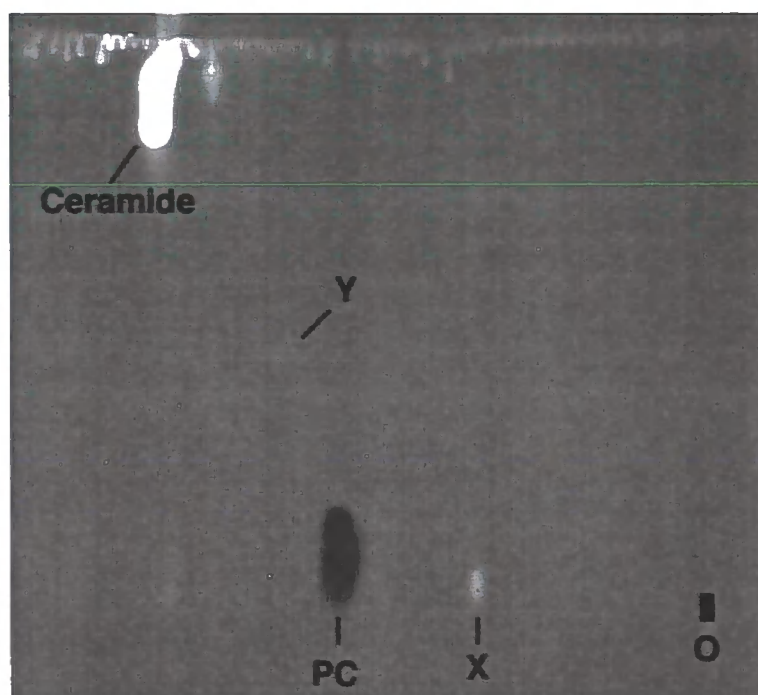


Figure 3.5:  
2D HPTLC analysis. *TbSLS 1000*- complemented cells were labeled with  $^{14}\text{C}$  choline (black) and BODIPY FL-ceramide (white).

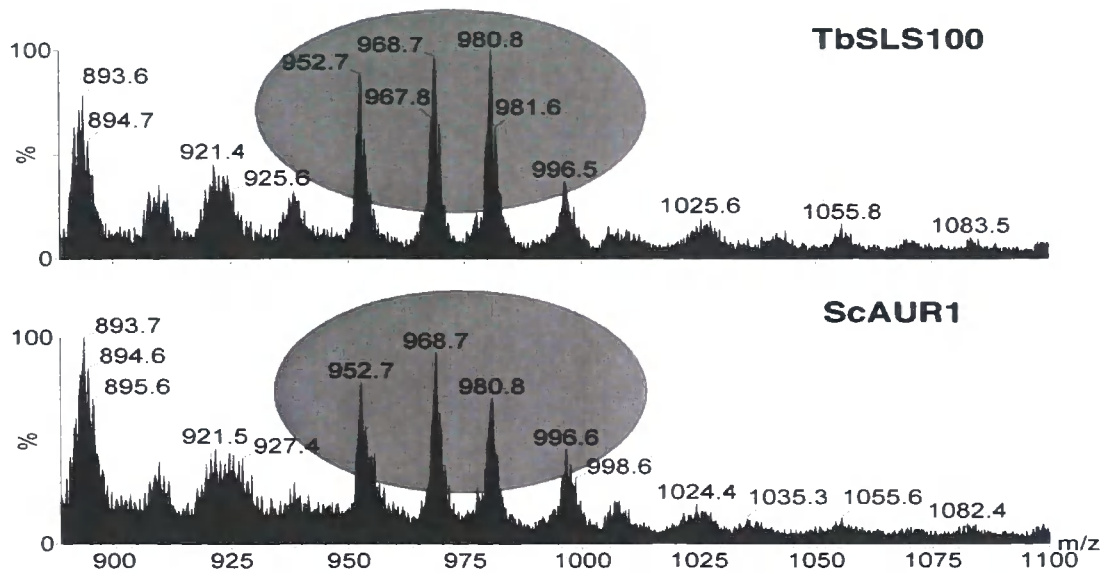


Figure 3.6:  
 Negative ion mass spectra of lipids from *TbSLS1000* and *ScAUR1*  
 complemented mutant yeast.

Moreover, lipids extracted from *TbSLS1000* were analysed by mass spectrometry (with Mike Ferguson, University of Dundee); 3 major complex sphingolipids were visualized in the negative ion spectrum at mass 952.7 (44:0,4), 968.7 (44:0,5) and 980.7 (46:0,4), all of these represent IPC species (figure 3.6). No SM or ethanolamine phosphorylceramide were detected (positive ion spectrum; data not shown). Comparing the mass spectrometry results of *TbSLS1000* and *ScAUR1* complemented yeast, showed no differences between the 2 cell lines. This indicated that *TbSLS1000* functions as an IPC synthase rather than SM synthase. However, this method did not identify the unknown X and Y species.

### 3.2.4 *in vitro* assay of *TbSLS1000*

In order to further understand the activity of *T. brucei* sphingolipid synthase, an *in vitro* assay was established as described in Materials and Methods. Microsomal membranes prepared from the *TbSLS1000* complemented yeast were treated with phosphatidylinositol (PI), phosphatidylethanolamine (PE) or phosphatidylcholine (PC) as donor substrates and BODIPY FL-ceramide as the receptor substrate.

Subsequently, the lipids were extracted and analyzed by HPTLC plate as previously. The addition of the donor substrate had no demonstrable effect on the high level of activity observed in the microsomes (data not shown). This might be due to the high level of donor substrate present in the microsomal membranes. In addition, when compared to metabolic labeling, less unknown Y was synthesized with respect to IPC in this assay.

Therefore, to reduce substrate contamination the microsomal membrane fractions were washed with 2.5% 3-[3-(cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) to eliminate most of the endogenous lipids and proteins (Aeed et al., 2004, Mina and Denny, personal communication).

Without the addition of donor substrate, and when compared with a crude preparation, the CHAPS-washed membranes showed a relatively low level of *TbSLS1000* activity as determined by the synthesis of unknowns X (figure 3.7). On the addition of exogenous substrate (bovine PI, PE or PC), only the sample which treated with PI showed an increase in sphingolipid synthase activity, as evidenced by the production of IPC. In contrast, equivalent experiments with *S. cerevisiae*, demonstrated that the yeast IPC synthase cannot use mammalian or plant PI. (Mina and Denny, personal communication) There was no difference in the formation of X or Y after adding

bovine PI in the *TbSLS1000* assay.

These results demonstrated that *TbSLS1000* functions as an IPC synthase indicating that cells produce IPC via IPC synthase. The identities of X and Y, products of both *T. brucei* BSF and *TbSLS1000* complemented yeast, are still unknown. However, it is assumed that they are the result of IPC synthase activity in *T. brucei* BSF cells.

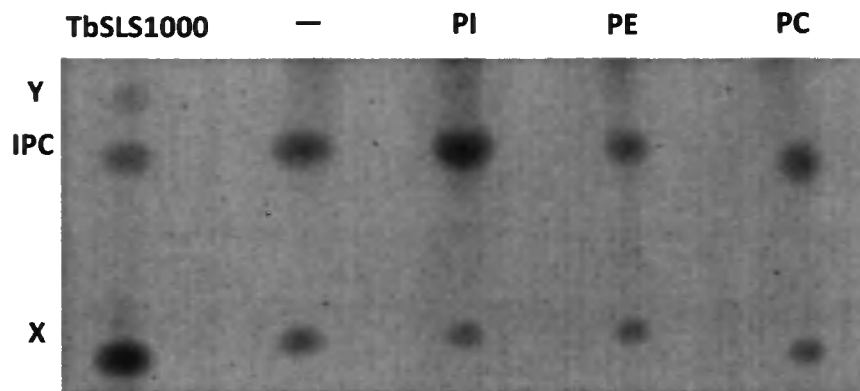


Figure 3.7:

*In vitro* assay of *TbSLS1000*. *TbSLS1000* – unwashed microsomal fraction assayed with BODIPY FL-ceramide as the receptor substrate and without exogenous donor substrate. CHAPS-washed microsomal fractions assayed with BODIPY FL-ceramide and either no exogenous donor substrate (-) or phosphatidylinositol (PI), phosphatidylethanolamine (PE) or phosphatidylcholine (PC).

### 3.2.5 Affect of temperature on *Tb*SLS1000 IPC synthase activity

In order to observe the effects of temperature on the *Tb*SLS1000, unwashed microsomes were incubated with BODIPY FL-ceramide (endogenous donor substrate acted as donor) at various temperatures (4°C, 20°C, 26°C, 30°C, 33°C, 37°C and 40°C).

As seen in figure 3.8, the activity of *Tb*SLS1000, as determined by the production of unknown X and IPC showed the lowest activity at 4°C and the highest activity at 40°C. Similarly, the *T. cruzi* IPC synthase activity had the lowest activity at 4°C, however the highest activity was observed at 37°C (Figueiredo et al., 2005). Patients infected by *T. brucei* usually have an acute disease with an accompanying high fever (Barret et al., 2003). In contrast, *T. cruzi* produce a chronic infection in tissues including cardiac system, digestive system and CSF without a significant fever (Barret et al., 2003). Additionally, this disease is transmitted by sand flies whose temperature reflects their tropical and sub-tropical habitat.

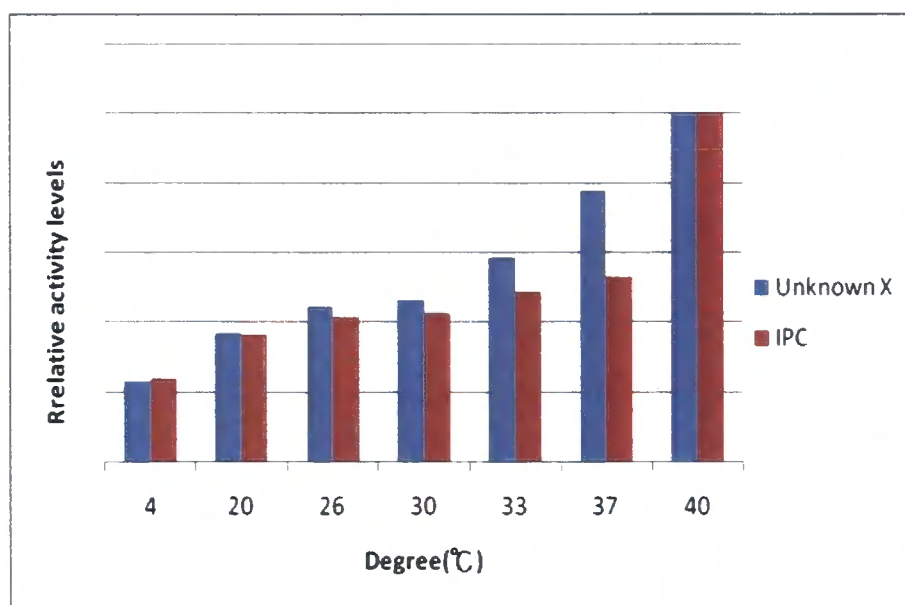


Figure 3.8:

Temperature assay. The established *in vitro* assay was performed at 4, 20, 26, 30, 33, 37 and 40°C and activity determined by the accumulation of products unknown X and inositol phosphorylceramide (IPC).

### 3.3 Aureobasidin A inhibition of *TbSLS1000*

#### 3.3.1 Agar diffusion assay

IPC synthesis a recognized target for anti-fungal drugs and aureobasidin A is widely utilized experimental inhibitor (Sugimoto et al., 2004). Aureobasidin A inhibits *Leishmania* *LmIPCS* activity (Denny et al. 2006; Mina and Denny, personal communication), therefore, it was investigated whether this drug affected the *LmIPCS* orthologue, *TbSLS1000*. Using the complemented yeast in an agar diffusion assay as described in Materials and Methods it was shown that *TbSLS1000* is sensitive to aureobasidin A at both 25 and 100  $\mu$ M. This demonstrated that the enzyme is more susceptible to the inhibitor than *LmIPCS* which is only sensitive to 100 $\mu$ M aureobasidin A (Denny et al., 2006). Cycloheximide (which inhibits protein translation) and myriocin (which inhibits serine palmitoyltransferase, an enzyme involved in the first step of sphingolipid synthesis) were used in the assay as positive controls.

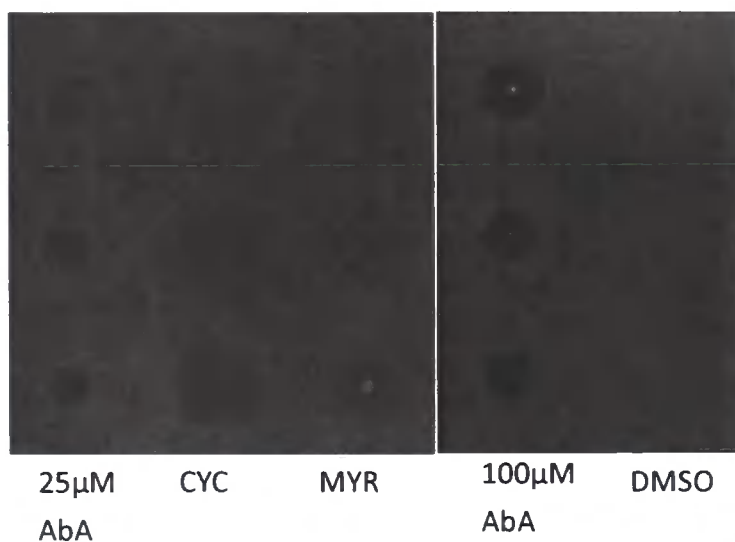


Figure 3.9:

Agar diffusion assay. *TbSLS1000* complemented yeast were grown in SD-HIS-URA agar and 1, 2 or 3  $\mu$ l of 25  $\mu$ M cycloheximide (CYC), 1 mM myriocin (MYR) and 25  $\mu$ M or 100  $\mu$ M of aureobasidin A (AbA) were dropped on to the plate. DMSO was used as a negative control.

### 3.3.2 *in vitro* assay of *TbSLS* aureobasidin A sensitivity

Having established that *TbSLS1000* is sensitive to aureobasidin A., unwashed *TbSLS1000* microsomal membranes were pretreated with 0-25nM of this drug and labeled with FL-ceramide. The lipids extracted fractionated by HPTLC and quantified to facilitate calculation of the IC<sub>50</sub> of aureobasidin A..

Figure 3.10 shows that the synthesis of IPC was strongly inhibited by a 25nM concentration of aureobasidin A. In contrast, the production of unknown X was less sensitive to the inhibitor. The IC<sub>50</sub> with respect to IPC synthase activity was calculated as approximately 15nM aureobasidin A. This contrasts with a values for the *S. cerevisiae* enzyme of 0.2nM (Nagiec et al., 1997).

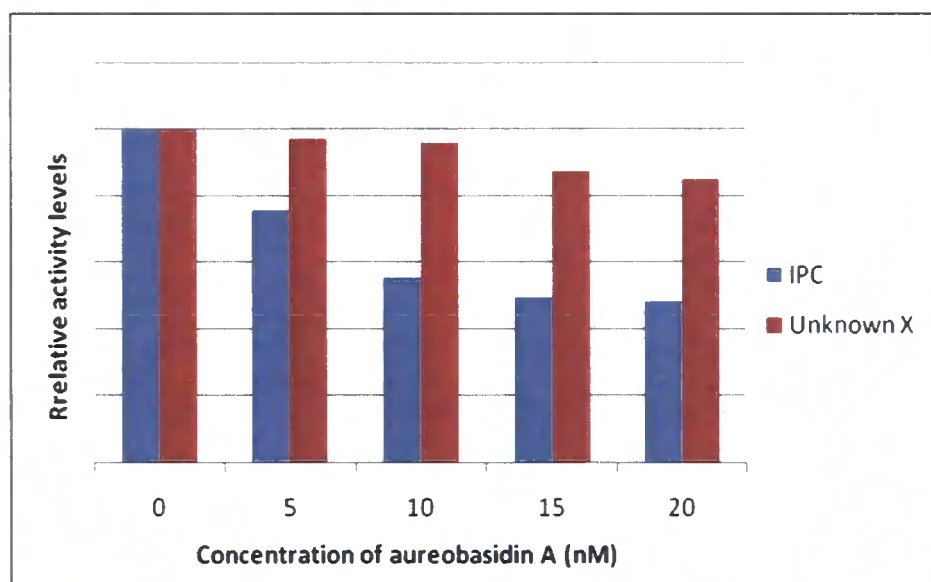


Figure 3.10:

The inhibitory effects of aureobasidin A in an *in vitro* assay of *TbSLS1000* activity. 0 to 20nM of aureobasidin A was added to each sample before the addition of BODIPY FL-ceramide.

### 3.3.3 Cell based assay of aureobasidin A sensitivity

It has been shown that aureobasidin A inhibits *TbSLS* in the complemented yeast cell line and *in vitro*. However, what is the effect of aureobasidin A on *T. brucei* BSF cells? To answer this question, the drug was added directly to the culture media at various concentrations and the cell growth assessed every 24 hours for 3 days.

As seen in figure 3.11, when the concentration of aureobasidin A was 5  $\mu\text{g}/\mu\text{l}$  or more, cell growth was completely inhibited and the parasites were dead within 24 hours (assessed by observing motility). The  $\text{IC}_{50}$  of *T. brucei* BSF cells was less than 1  $\mu\text{g}/\mu\text{l}$  or 0.9mM aureobasidin A (figure 3.11).

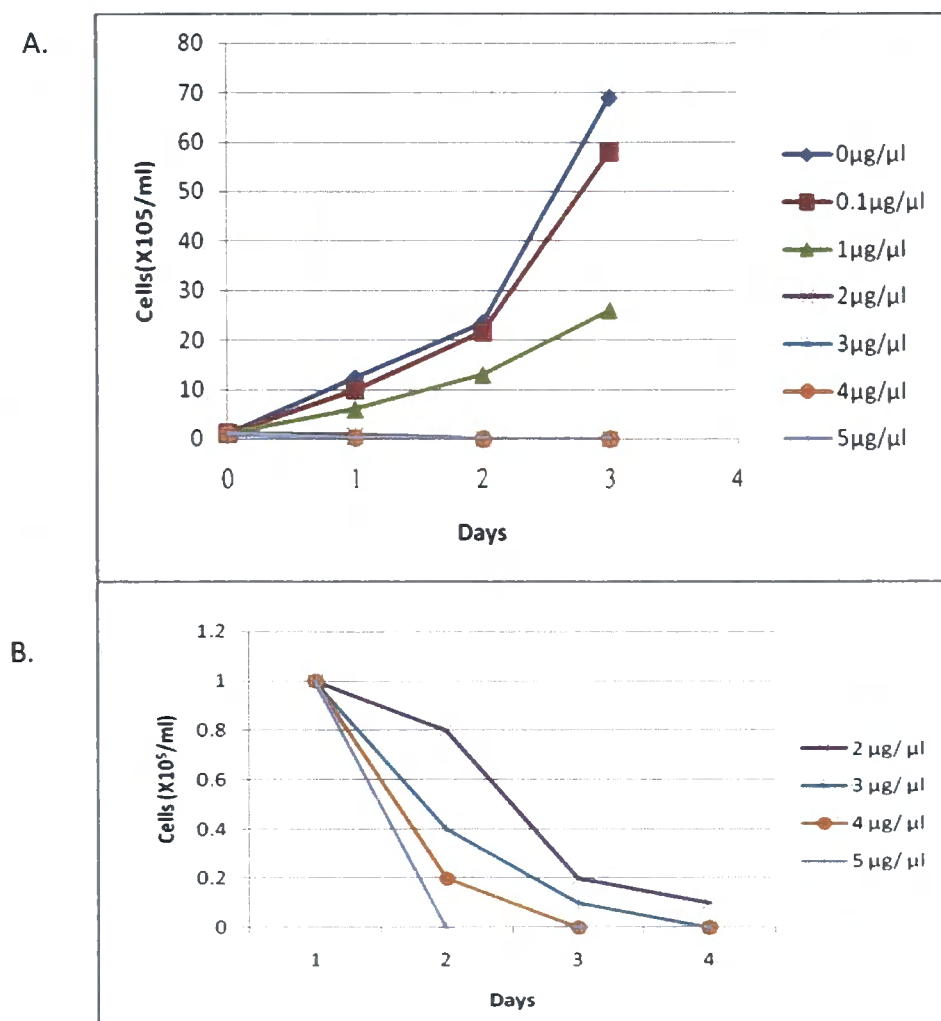


Figure 3.11: *T. brucei* BSF cell growth curve. Aureobasidin A at 0 to 5  $\mu\text{g}/\mu\text{l}$  was added to the growth media. A: 0-5  $\mu\text{g}/\mu\text{l}$ . B: 2-5  $\mu\text{g}/\mu\text{l}$ .



In order to analyze the effect of aureobasidin A on sphingolipids synthase, *T. brucei* BSF parasites were treated with aureobasidin A between 0 and 4  $\mu\text{g}/\mu\text{l}$  and incubated at 37°C for 2 hours (before the cells started dying) and the parasites were labeled with BODIPY FL-ceramide. The lipids were extracted and fractionated by HPTLC (figure 3.12). The activity of *TbSLS*, determined by the synthesis of both unknowns X and Y, decreased with increasing concentrations of aureobasidin A which suggested that inhibition of *TbSLS* by aureobasidin A leads to the death of *T. brucei* BSF cells.

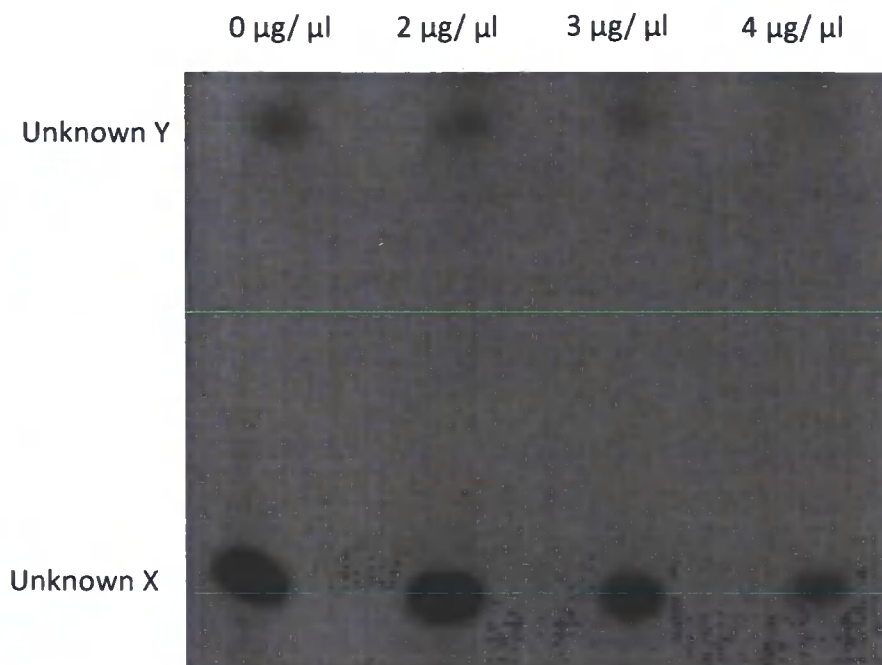


Figure 3.12:  
Metabolic labelling of *T. brucei* BSF parasites with BODIPY FL-ceramide in the presence of 0-4  $\mu\text{g}/\mu\text{l}$  of aureobasidin A.

## 3.4 RNAi

### 3.4.1 RNAi constructs

Three RNAi constructs were formed in the p2T7 vector, which is designed to produce double stranded RNA from head-to-head T7 promoters when induced by tetracycline (LaCount et al. 2000), and used to inhibit the expression of *T. brucei* genes involved in sphingolipid biosynthesis. The first of these was *Tb-LCB2* (*Tb10.70.3220*) which encodes part of the SPT, the first enzyme in sphingolipid synthesis.

The other 2 constructs were based on sequence common to all 4 *TbSLS* isoforms (*Tb09.211.1000*, 1010, 1020 and 1030): *TbSLS* RNAi1 and *TbSLS* RNAi2. These constructs were linearised and transfected into SMB (Single Marker Bloodstream-form) *T. brucei* (Wirtz et al., 1999) which were then selected with phleomycin.

### 3.4.2 *Tb-LCB2* RNAi

Non-transfected SMB cells were treated with doxycyclin as a control. Doxycyclin is a tetracycline derivative which is used to induce the production of RNAi. As can be seen in figure 3.13, the SMB cells grew normally in the presence of doxycyclin, this indicates that the antibiotic has no toxic affect.

However, on the induction of *Tb-LCB2* RNAi the growth rate became decreased markedly and cell death was apparent (by light microscopy). This indicated that sphingolipid biosynthesis is essential for the proliferation of *T. brucei* BSFs. After subculturing the cells twice, the growth rate of the induced cells was equivalent to that of the non-induced cells, indicating that parasites resistant to RNAi had been selected (figure 3.14).

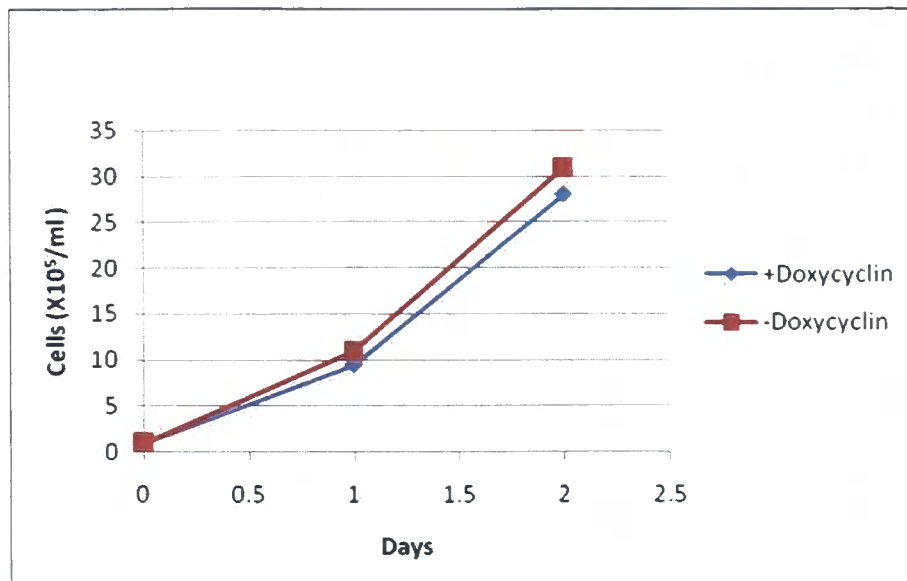


Figure 3.13:  
Growth curve of control SMB parasites with and without doxycyclin.

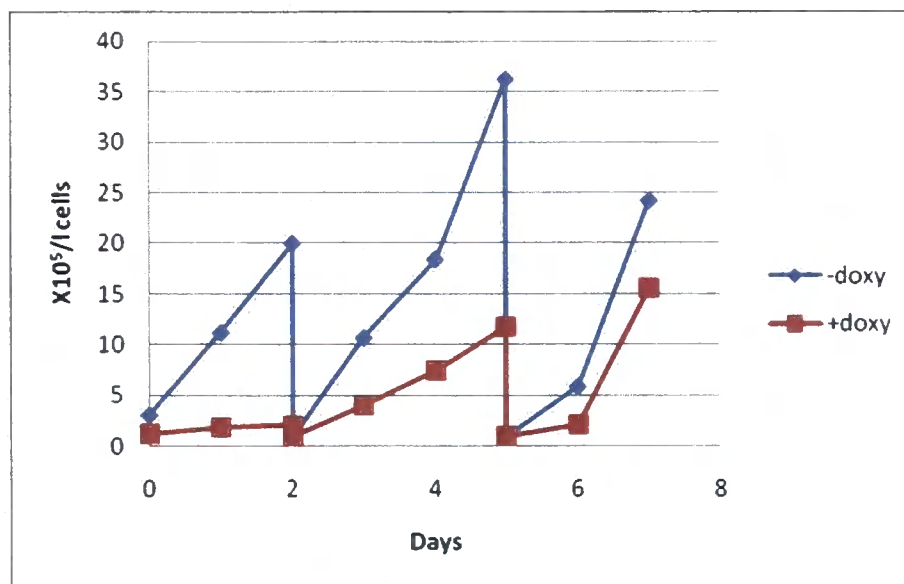


Figure 3.14:  
Growth curve of transfected LCB2 RNAi cells induced by doxycyclin.  
Parasites were subcultured to  $1 \times 10^5$ /ml when non-induced cells reached or exceeded  $2 \times 10^6$ /ml.

In order to confirm that this phenomenon was due to the specific suppression of the target gene by RNAi, RNA was extracted from the cells after 2 days of induction and analyzed by RT-PCR. *Tb*-LCB2 mRNA was clearly decreased after 48 hours induction, whereas control-  $\alpha$ -tubulin mRNA was unaffected. This indicated that the RNAi response was gene specific.

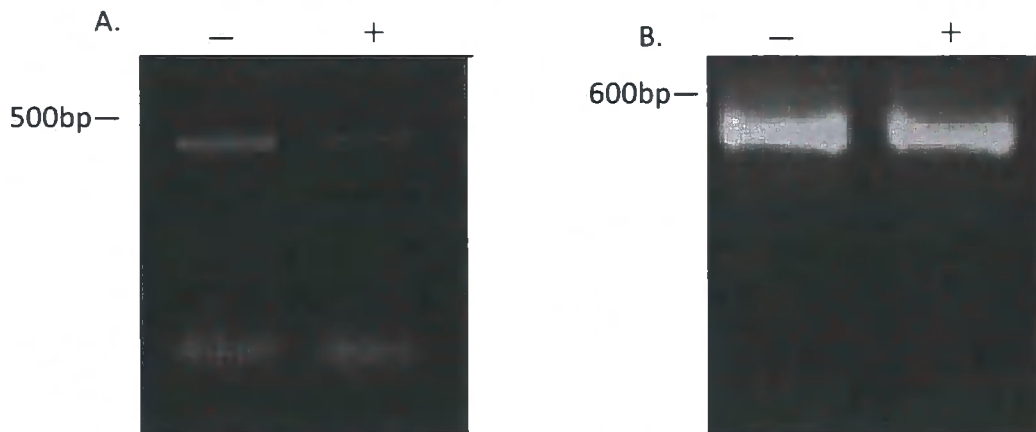


Figure 3.15:

RT-PCR of *T. brucei* LCB2RNAi cells.

A. LCB2 mRNA fragment (~480bp), B.  $\alpha$ -tubulin mRNA fragment (~580bp),

—: without doxycyclin, +: with doxycyclin for 48 hours

### 3.4.3 *Tb*SLS RNAi

RNAi constructs, *Tb*SLS RNAi1 and *Tb*SLS RNAi2, were transfected into *T. brucei* SMB cells. However, only *Tb*SLS RNAi2 allowed selection of viable parasites.

Non-induced *Tb*SLS RNAi2 cells grew in a similar manner to the parental SMB parasites. In contrast, the induced parasites grew very slowly with significant cell death (by light microscopy). Just like *Tb*LCB2 RNAi, the growth rate of induced cells was equivalent to that of non-induced cells after subculturing the cells twice (figure 3.16).

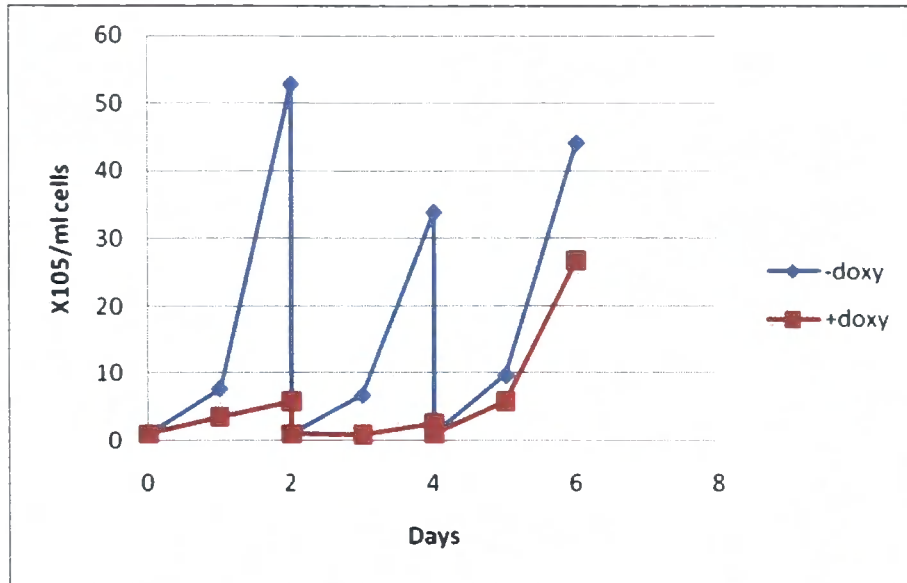


Figure 3.16:

Growth curve of transfected *TbSLS* RNAi cells induced by doxycyclin. Parasites were subcultured to  $1 \times 10^5$ /ml when non-induced cells reached or exceeded  $2 \times 10^6$ /ml.

Again, RT-PCR was used to confirm the specificity of the RNAi. As shown in figure 3.17, *TbSLS* mRNA was significantly decreased after 48 hours induction. Control  $\alpha$ -tubulin mRNA was unaffected.

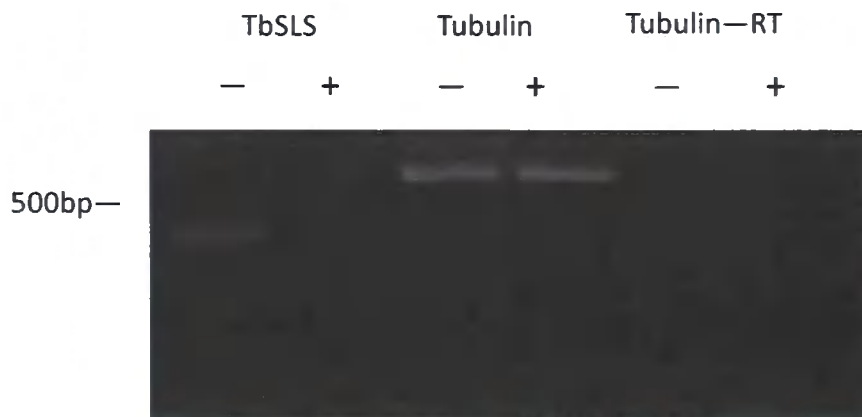


Figure 3.17:

RT-PCR of *T. brucei* SLS RNAi cells.

A. *TbSLS* mRNA fragment (~400bp),

B.  $\alpha$ -tubulin mRNA fragment (~580bp),

—: without doxycyclin, +: with doxycyclin for 48 hours

Negative control is tubulin RT-PCR without RT

To directly ascertain the affect of *TbSLS* RNAi2, induced and non-induced cells were grown for 3 days. After that, cells were labeled with BODIPY FL-ceramide and analysed as described previously. After induction, the *TbSLS* RNAi2 transfected cells produced 38% less of X than non-induced cells. However, there was no difference in the quality of Y (data not shown). The reason of this is still unknown.

All of these data confirm that sphingolipid synthesis is essential for the proliferation of BSF and procyclic *T. brucei* (Sutterwala et al., 2007; Fridberg et al., 2008). They also demonstrated that the *T. brucei* sphingolipids synthase (*TbSLS*) is an essential activity in the parasite and a valid drug target.

# **Chapter 4:**

# **Discussion**

## Chapter 4: Discussion

### 4.1 Identification of the *T. brucei* sphingolipid synthase

In recent years, it has become clear that sphingolipid synthase represents a promising drug target for many parasitic diseases, such as those caused by *T. cruzi* and *L. major* (Heung et al., 2006). However, the biochemical function of the *T. brucei* sphingolipid synthase remained unknown (Denny et al., 2006). To begin to understand the role of this enzyme in pathogenesis, bloodstream form cells were labeled with the fluorescent substrate- FL-ceramide and lipids extracted and analyzed by HPTLC.

Two species of complex sphingolipid were synthesized and evident on the plate: SM (X) and Y (figure 3.2). Comparing the R<sub>f</sub> values, under the conditions employed, of labeled *T. brucei* sphingolipid species with similarly labeled *S. cerevisiae* (which synthesize IPC via IPC synthase, Becker and Lester, 1980) and CHO cells (which produce SM via SM synthase, Huiterna et al., 2004), X (0.45) migrated with SM (0.45) and Y(0.78) migrated further than IPC (0.74).

To analyse the *T. brucei* sphingolipid synthase, one of the four coding sequences in genome database ([www.genedb.org](http://www.genedb.org)), *Tb09.211.1000* (*TbSLS1000*), was cloned into the yeast expression vector pRS426MET25 subsequently transformed into YPH499-HIS-GAL-AUR1 *S. cerevisiae* mutant yeast in which the essential AUR1, the IPC synthase is regulated by GAL1 promoter and so repressed in the presence of glucose (Denny et al., 2006). *TbSLS1000* complemented the auxotrophic mutant yeast and metabolic labeling with FL-ceramide and analyses of these and *T. brucei* bloodstream form cells found that whilst the parasite produced X and Y, the complemented yeast produced lipids that co-migrated with X, Y and IPC (figure 3.2).

To identify X and Y, *TbSLS1000* complemented yeast were labeled with C<sup>14</sup> choline



which would be incorporated into SM and analyzed by 2D-HPTLC, in addition mass spectrometry was employed. These experiments failed to identify X or Y, however, mass spectrometry confirmed the presence of IPC. This indicated that *TbSLS* functions primarily as an IPC synthase in this axenic system.

To analyse the *T. brucei* sphingolipid synthase activity *in vitro*, detergent-washed *TbSLS1000* complemented yeast microsomal fractions were treated with FL-ceramide as acceptor substrate and PI, PE or PC as donor substrates for IPC, EPC and SM synthesis respectively. The result shown in figure 3.7 demonstrated that the sample treated with PI significantly increased the synthesis of IPC. PE and PC did not function as substrates. This confirmed that *TbSLS1000* functions as an IPC synthase.

Furthermore, the microsomal membranes were reacted at different temperatures to observe the variation of sphingolipid synthase activity. As seen in figure 3.6, *TbSLS1000* had highest activity at 40°C, whilst *T. cruzi* sphingolipid synthase has highest activity at 37°C (Figueiredo et al., 2005) and the *L. major* enzyme at 30°C (J. Mina, personal communication). Unlike *L. major*, which causes dermal lesions where the temperature is near 30-33°C (<http://www.who.int/leishmaniasis/en/>), or *T. cruzi*, which leads in chronic disease of cardiac tissue, patients infected by *T. brucei* usually have an acute disease with an accompanying high fever (Barret et al., 2003). These results reflect the different pathogenic lifestyle of these parasites. Therefore, the different optimal operating temperatures of these orthologues may reflect the different pathogenic lifestyle of these parasites.

## 4.2 Inhibition of *T. brucei* SLS by aureobasidin A

Having established that *TbSLS1000* functions as an IPC synthase, subsequent studies focused on the inhibition of this activity. Aureobasidin A (AbA), which is non-toxic to animals, has been used as a specific, experimental anti-fungal to inhibit IPC synthase in yeast species, such as *S. cerevisiae* and *Candida* (Zhong et al., 2000). Experiments have also demonstrated that AbA inhibits IPC synthase in the kinetoplastid parasites *T. cruzi* and *L. major*. (Figueiredo et al., 2005, Denny et al., 2006)

To establish whether AbA inhibits the *T. brucei* sphingolipids synthase, *TbSLS1000* complemented yeast were assayed for their susceptibility to this inhibitor in an agar diffusion assay. Cells growth was inhibited by 25 $\mu$ M and 100 $\mu$ M AbA indicating that *T. brucei* sphingolipid synthase is susceptible to AbA. Previously, *LmIPCS* complemented yeast were shown to only be susceptible to the higher concentration of AbA (Denny et al., 2006). Metabolic labeling with FL-ceramide demonstrated that the synthesis of labeled X and IPC decreased with increasing the concentrations of AbA and Y was barely detectable after treatment. The activity of *TbSLS1000* (as defined by the synthesis of X, Y and IPC) was totally inhibited when the cells were treated with more than 25nM AbA. The IC<sub>50</sub> with respect to IPC synthase activity was calculated as approximately 15nM AbA demonstrating that *TbSLS1000* activity is very sensitive to this inhibitor.

Despite these *in vitro* analyses the efficacy of Ab A with respect *T. brucei* remained unknown. To begin to understand the effect of this drug on pathogenic bloodstream form *T. brucei*, 0-5 $\mu$ g/ $\mu$ l of AbA was added to cells and the parasites observed by light microscopy every 24 hours. As expected, the parasites grew more slowly and died in the presence of AbA.

To verify the effect of AbA inhibition on the parasites, *T. brucei* bloodstream form cells were labeled with FL-ceramide and lipids extracted to analyze the variation of *TbSLS* activity after treated with AbA 2 hours. Notably, the quantities of X and Y decreased which indicated that the inhibition of the enzyme by AbA lead to growth inhibition and death of *T. brucei* bloodstream form parasites.

### **4.3 Inhibition of *T. brucei* SLS by RNAi**

Following the chemical inhibition of *TbSLS*, RNAi of all 4 isoforms simultaneously was also used to genetically inhibit the function of the *T. brucei* enzyme.

On induction of RNAi, parasites ceased to grow and many were scored as dead by light microscopy. RT-PCR confirmed that this was attributable to the specific down-regulation of *TbSLS* mRNA. As expected, RNAi was *TbSLS* lead to a reduction in induced cells production of sphingolipid X compared with controls after FL-ceramide labeling. However, not much difference in the production of enzyme product Y. Given that the target gene was not fully suppressed is the residual activity maybe due to extant enzyme activity.

#### **4.4 Conclusion**

We have demonstrated that the *T. brucei* sphingolipid synthase functions as an IPC synthase. Furthermore, this enzyme is shown both chemically and genetically to be essential for *T. brucei* bloodstream form growth, validating *TbSLS* as a drug target in the pathogenic stages of this parasite.

However, since only one of the 4 *TbSLS* coding sequences (*Tb09.211.1000*) was amplified and analysed in this study, it will be necessary to analyse the other 3 closely related isoforms to identify any differences.

Moreover, the mechanism of *T. brucei* sphingolipid synthase activity is still unclear. *T. brucei* bloodstream form cells produce X and Y rather than IPC as would be expected from the analysis here. Further analysis to identify sphingolipids X and Y is necessary to fully understand the function of *T. brucei* sphingolipid synthase.

Ultimately it is hoped that these studies may lead to the development of a new drug leads and, subsequently, to therapies. Such drugs are much needed due to the toxicity of current treatments for African sleeping sickness and because of the rise drug resistance was found in this disease. (Mäser et al., 2003)

#### **4.5 Future work**

It remains necessary to identify the unknown sphingolipids X and Y. Recent work has demonstrated that X is sensitive to SMase, indicating that it is SM (Pan et al, submitted). However, mass spectrometry failed to identify SM (or other non-inositol sphingolipids, i.e. Y) in extracts of complemented yeast. An alternative approach would be to use this technique to directly detect labeled products as previously described (Landoni et al, 2008). This could be performed on labeled microsomal samples or metabolically-labeled yeast.

The experiments described in this report were focused on *in vitro* studies. Subsequent research will need to focus on the the function of *T. brucei* SLS *in vivo*. To facilitate this, the established mouse animal model would be utilized. Mice would be infected with bloodstream form RNAi transfected parasites by intra-peritoneal inoculation. Half of the infected mice would be given water containing doxycycline (which will induce RNAi in the *T. brucei*) and the survival rate of these mice, compared with controls, established (Lecordior et al., 2005). In addition, the efficacy of AbA in infected animals is unknown, although this drug has previously been show to be effective against systemic *Candida albicans* in a mouse model when given both orally and sub-cutaneously (Takesako et al, 1993). Similar experiments with mice infected with *T. brucei* as above could establish the efficacy of AbA against an animal model of African Sleeping Sickness (Yabu et al., 2003).

The primary future objective must be the discovery of more lead inhibitors of *T. brucei* SLS. The development of an *in vitro* microtitre plate-based assay in the laboratory makes high throughput screening against this enzyme activity a possibility (Mina et al, 2009). This may identify compounds which could then further chemically modified to increase efficacy before being tested *in vitro* and *in vivo*, as discussed above, against the laboratory isolate used here and against field isolates to confirm effectiveness.

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# **Appendix**



# **Targeting the *Trypanosma brucei***

## **sphingolipid synthase, an essential enzyme**

Pan, S. Y.<sup>1,+</sup>, Mina, J.G.<sup>1,2,+</sup>, Wansadhipathi, N.K.<sup>1</sup>, Shams-Eldin H.<sup>3</sup>,

Schwarz, R.T.<sup>3</sup>, Steel, P.G.<sup>2</sup> and Denny, P.W.<sup>1,2,\*</sup>

**<sup>1</sup>Infectious Diseases Group, Wolfson Research Institute, Durham**

**University, Queen's Campus, Stockton-on-Tees, TS17 6BH, UK**

<sup>2</sup>Centre for Bioactive Chemistry, Department of Chemistry,

Durham University, Durham, DH1 3LE, UK

<sup>3</sup>Institut für Virologie, Zentrum für Hygiene und Infektionsbiologie,

Philipps-University Marburg, Hans-Meerwein-Strasse, 35043 Marburg, Deutschland; and

Unité de Glycobiologie Structurale et Fonctionnelle, UMR CNRS/USTL n° 8576 - IFR 118,

Université des Sciences et Technologies de Lille, 59655 Villeneuve D'Ascq cedex – France.

+These authors contributed equally to this work

\*Address correspondence to: Dr Paul W. Denny, Infectious Diseases Group,

Wolfson Research Institute, Durham University,

Queen's Campus, Stockton-on-Tees, TS17 6BH, UK.

Tel. +44 (0)191 334 0319; Email: <mailto:p.w.denny@durham.ac.uk>

**Running title:** A novel drug target for African Trypanosomiasis

## **Abstract**

Sphingolipids are important components of eukaryotic membranes, particularly the plasma membrane, and are involved in a diverse array of signal transduction processes. In the Eukaryota the biosynthetic pathway for the formation of these lipid species is largely conserved. However, several pathogenic fungi and protozoa synthesize inositol phosphorylceramide (IPC) as the primary phosphosphingolipid rather than sphingomyelin (SM) as do mammals. This process is catalyzed by the enzyme IPC synthase, a recognized target for anti-fungals encoded by the *AUR1* gene in yeast. Recently functional orthologues of the AUR1p have been identified in a group of insect vector-borne pathogenic protozoa, the Kinetoplastida, which are responsible for a range of so-called neglected diseases. Of these the *Trypanosoma brucei* species are the causative agents of Human African Sleeping Sickness in many of the most underdeveloped regions of Africa. The available treatments for these diseases are limited, of decreasing efficacy, and often demonstrate severe side-effects. Against this background the *T. brucei* sphingolipid synthase, an orthologue of the yeast AUR1p, may represent a promising target for novel anti-protozoals. Our studies identify this protein as a novel bifunctional enzyme capable of catalysing the synthesis of both IPC and SM, both known to be present in the parasite. Furthermore, the synthase is essential for parasite growth and can be inhibited by a known anti-fungal at sub-nanomolar levels *in vitro*. Most notably this drug demonstrates trypanocidal activity against cultured bloodstream form parasites. Thus *T. brucei* sphingolipid synthase represents a valid and promising drug target.

## Introduction

*Trypanosoma brucei* species are protozoan parasites of the order Kinetoplastidae and the etiological agents of both Human African Trypanomiasis (HAT), otherwise known as sleeping sickness, and diseases of economically important animals (e.g. nagana in cattle) (1). These diseases are endemic in much of sub-Saharan Africa, with HAT causing a burden of approximately 1.6 million disability adjusted life years (<http://www.who.int/tdr/>). This distribution across some of the most under developed regions of the world is coupled with a paucity of effective therapies with those available being either too expensive (e.g. eflornithine) or exhibiting catastrophic side-effects (e.g. melarsoprol). Together with the leishmaniasis (caused by the related *Leishmania* species), HAT is described by the World Health Organization as a Category I – Emerging or uncontrolled disease (<http://www.who.int/tdr/>). This categorization emphasizes the importance of the discovery of new drug targets and anti-HAT compounds to combat a disease which causes in excess of 50 000 deaths per annum (25).

Sphingolipids are a diverse group of amphipathic lipids that perform essential functions in eukaryotes. For example, the unmodified, base sphingolipid ceramide acts as a secondary signalling molecule (11) and more complex species are implicated in the formation and function of signal transduction complexes (20, 24). The primary phosphosphingolipid species in mammalian species, including humans, is sphingomyelin (SM). SM is formed by the transfer of the phosphorylcholine head group from phospholipid phosphatidylcholine (PC) to ceramide, a reaction catalyzed by SM synthase (15). Unlike mammals fungi, plants and at least some protozoa produce inositol phosphorylceramide (IPC) as their primary phosphosphingolipid (18). In these organisms IPC synthase catalyzes the transfer of phosphorylinositol from

phosphatidylinositol (PI) to ceramide (2, 5, 7). IPC synthase has long been established and studied as a target for novel anti-fungals (12, 21). More recently this enzyme activity has come under scrutiny as a potential target for anti-protozoals (31). With the recent identification and characterization of the *Leishmania* IPC synthase (7) it has become possible both to validate this activity as a drug target and, furthermore, begin to investigate the specific efficacy of known fungal IPC synthase inhibitors, and novel compounds, against the enzyme and the pathogen itself. Four closely related orthologues of *Leishmania* IPC synthase are apparent in the *T. brucei* database (7), and mass spectrometry of isolated fractions has revealed that whilst the predominant phosphosphingolipid in pathogenic bloodstream form parasites is SM; in insect stage, procyclic *T. brucei*, the primary species is, as in *Leishmania*, IPC (13). Here we describe the characterization of the model African trypanosome, *T. b. brucei*, sphingolipid synthase (TbSLS) which demonstrates itself to be a novel bi-functional enzyme with the ability to catalyze the biosynthesis of both IPC and SM, thus reflecting the sphingolipid profile of the parasite. Importantly, the IPC synthase activity of TbSLS is acutely sensitive to the well characterized specific fungal inhibitor aureobasidin A (12, 21). Furthermore, bloodstream form *T. brucei* are rapidly killed at sub-micromolar concentrations of this drug. Down-regulation of TbSLS, using inhibition RNA (RNAi), in bloodstream form parasites demonstrated that the enzyme activity is essential for growth thus validating this enzyme as a target for the development of new anti-HAT therapies.

## Materials and Methods

### *Sequence analyses*

Sequence alignments were made using CLUSTALW (16). Topology predictions were performing using the PHD package (28).

### *Functional identification of Trypanosoma brucei synthase (TbSLS)*

TbSLS1 (Tb09211.1000) and TbSLS4 (Tb09211.1030) were amplified with *Pfu* polymerase (Promega) from genomic DNA from *T. b. brucei* strain Lister 427 using primer pairs (homologous sequence underlined):

TbSLS1 – CCGGAATTCATGATTAGTTACCCTTTCTTCTCCC and

CCGCTCGAGTCACACATACGCCCCACATTTAAAC

TbSLS4 - CCGGAATTCATGATTAGTTACCCTTTCTTCTCCC and

CCGCTCGAGTCATACCTCGTTAGTTGATAC

The PCR products were subsequently cloned into the yeast expression vector pRS426 MET to give pRS426 TbSLS1 and pRS426 TbSLS4. These, together with pRS426 ScAUR1 were used to transform the *YPH499-HIS-GAL-AUR1 S. cerevisiae* strain (7), and functionally complemented transformants selected on non-permissive SD medium (0.17% Bacto yeast nitrogen base, 0.5% ammonium sulphate and 2% dextrose) containing the nutritional supplements to allow selection of transformants.

### *Metabolic labelling and analyses*

Logarithmic phase yeast were harvested and incubated at 10 OD<sub>600</sub>/ml in 0.25 ml SD medium at 30°C for 30 minutes. The cells were subsequently incubated in SD supplemented with 5 µM of NBD C<sub>6</sub>-ceramide (Invitrogen) conjugated to fat-depleted

bovine serum albumen (Sigma-Aldrich) for 60 minutes. Cells were harvested by centrifugation and washed twice with phosphate buffered saline. Chloroform/methanol (0.4 ml; 1:1 v/v) was added and cells were disintegrated with glass beads. The pellet was re-extracted several times with chloroform/methanol/water (10:10:3). The reaction products were fractionated using HPTLC silica plates (Merck) and eluent system Chloroform:Methanol:aqueous 0.25% KCl (55:45:10). Imaging and quantification was carried out using a FLA3000 scanner (Fujifilm) and AIDA Image Analyzer® software (version 1.3). Vero cells and untransformed *Saccharomyces cerevisiae* were labeled and processed for use as controls. Sphingomyelinase (Sigma Aldrich) was used to identify sphingomyelin in the NBD C<sub>6</sub>-ceramide lipids extracted from the complemented yeast as previously described (30).

#### *In vitro assay of TbSLS1 activity*

Microsomal membranes from exponentially growing *YPH499-HIS-GAL-AURI* pRS246 TbSLS1 were prepared as previously described (9) and the isolated membrane fraction re-suspended in storage buffer (50 mM Tris/HCl pH 7.4, 20% (v/v) glycerol, 5 mM MgCl<sub>2</sub>) with Complete® EDTA-free Protease Inhibitor Cocktail (Roche Applied Science). The protein content was determined using a Bradford assay (Brilliant Blue G-250, BioRad) and the concentration adjusted to adjusted to 10 mg/ml. The microsomal membranes were washed in 2.5% CHAPS (v/v; Sigma Aldrich; 4 °C, 60 minutes), isolated by centrifugation (150000 g, 4 °C and 90 min), re-suspended in storage buffer at 10 mg/ml and stored at -80 °C until use.

The assay mix contained 1 mM donor substrate (bovine liver PI, PC or PE, Avanti Polar Lipids), 100 mM Tris HCl, 10 mM EDTA, 6 mg/ml BSA and 200 μM NBD C<sub>6</sub>-ceramide (8). The reaction was incubated at 30 °C for 60 minutes then quenched by the addition of 150 μl of Chloroform:Methanol:Water (10:10:3). After

biphasic separation the organic layer was removed dried in a rotavapor (Eppendorf Concentrator 5301) and resuspended in 20  $\mu$ l of 10:10:3 and analysed as above.

For inhibition experiments the reaction mix was pre-incubated for 30 minutes with appropriate quantities of aureobasidin A (Takara) before the addition of NBD C<sub>6</sub>-ceramide.

#### *Agar diffusion assay*

Wild type YPH499 yeast and the transgenic strain *YPH499-HIS-GAL-AURI* complemented with ScIPCS or TbSLS were assayed for susceptibility to aureobasidin A (Takara), myriocin (Sigma Aldrich) and cycloheximide (Sigma Aldrich) as previously described (7). Briefly,  $2.4 \times 10^7$  logarithmically dividing cells were embedded in 15 ml of YPD agarose (2% dextrose, 1% yeast extract, 2% peptone, 0.8% agarose) on 100mm<sup>2</sup> square Petri dishes (Sarstedt). Inhibitors were applied in DMSO at the concentrations described below and the dishes incubated at 30°C.

#### *Parasite culture*

Bloodstream form *T. brucei* strains Lister 427 and its engineered variant, Single Marker Bloodstream (SMB, *T7RNAP::TETR::NEO*; 33) were maintained *in vitro* at 37°C with 5% CO<sub>2</sub> in HMI-9 medium supplemented with 10% FCS and, for SMB, 2.5  $\mu$ g/ml G418.

#### *Inhibition RNA (RNAi) of TbSLS*

An 165 base pair sequence fragment common to all four TbSLS open reading frames was amplified from genomic DNA using *Pfu* polymerase and primer pair (homologous sequence underlined): CATAGATCTAGAGGGTCCATACACTGTG and CATAGATCTAGACGAGAGGCAACGATGC

This PCR product was then cloned into the RNAi vector p2T7 (17), and following linearization, 10 µl transfected into SMB *T. brucei* and transformants selected using 2 µg/ml phleomycin (Sigma Aldrich). Following induction with 1 µg/ml doxycycline cell growth and viability were determined at 24 hour intervals by light microscopy using an Improved Neubauer Haemocytometer.

48 hours post-induction total RNA was isolated (RNeasy, Qiagen) and RT-PCR performed (SuperScript II, Invitrogen) using the primer pairs:

TbSLS: AACTGTACCTTCTTCACCG and CGAGAGGCAACGATGC

Tb β tubulin: GGAGCGCATCAATGTGTAC and CAGGCAGCAGGTGACGCCG

*T. b. brucei susceptibility to aureobasidin A*

*T. b. brucei* Lister 427 were cultured in the presence of various concentrations of aureobasidin A. Growth and viability was analysed at 24 hour intervals as above.



## Results

### *Identification and characterisation of the T. brucei sphingolipid synthase*

Four gene sequences (Tb09211.1000, Tb09.211.1010, Tb09.211.1020 and Tb09.211.1030) were previously identified in the genome database ([www.genedb.org](http://www.genedb.org)) as sequence orthologues of the inositol phosphorylceramide synthase (LmIPCS) from the related kinetoplastid parasite, *Leishmania* (7). Like LmIPCS, TbSLS possesses conserved sphingomyelin (SM) synthase domains: D1, D3 and D4 (7). D3 and D4 are homologous to the C2 and C3 motifs in lipophosphate phosphatases (LPP) (22) and domains 3 and 4 of the fungal AUR1p (14). D3 and D4 contain the histidine and aspartate residues that form the catalytic triad that mediates nucleophilic attack on lipid phosphate ester bonds (15, 22); site directed mutation of this triad inhibits both fungal and *Leishmania* IPC synthase (19); Mina and Denny, unpublished data). Further analysis of the *T. brucei* genomic sequence identified the complete open reading frames found on chromosome 9 which are predicted to encode four trans-membrane proteins with more than 90% identity and 94% similarity (figure 1). Most variation occurs at the carboxy-termini, a region predicted to lie on the cytosolic side of the membrane away from the active site at the Golgi lumen (7) with another variable domain close to the second predicted trans-membrane domain. One of the predicted *T. brucei* sphingolipid synthase isoforms (Tb09.211.1000; TbSLS1) was further analysed in this study. However, the most distant isoform from TbSLS1 (Tb09.211.1030; TbSLS4) with respect to the internal variable domain was also subjected to preliminary analyses.

The auxotrophic mutant *Saccharomyces cerevisiae* strain, YPH499-HIS-GAL-AUR1, in which the essential AUR1 IPC synthase gene is under the control of a galactose inducible promoter is unable to grow in the absence of galactose or the presence of the

repressor glucose, a phenotype that can be rescued by the expression of LmIPCS (7). Similarly, TbSLS1 expression complemented the AUR1 mutant yeast line indicating that it too is a functional orthologue of the yeast AUR1 gene (figure 2). TbSLS4 similarly complemented this mutant line (data not shown).

To begin to understand the function of the *T. brucei* sphingolipid synthase, the auxotrophic YPH499-HIS-GAL-AUR1 yeast complemented with TbSLS1 or TbSLS4 were metabolically labelled with fluorescent NBD C<sub>6</sub>-ceramide (a substrate for sphingolipid synthases, including those from the kinetoplastids (7)). Under these conditions *S. cerevisiae* synthesizes a single labelled species, IPC (7). In contrast, both TbSLS1 and TbSLS4 are shown to mediate the synthesis of two lipid species: one comigrated with a SM standard, the other co-migrated with an IPC standard (figure 3A). TbSLS1 was chosen for further study due to the predominance of the SM-like species in the complemented yeast, SM is known to be present in pathogenic bloodstream form *T. brucei* whilst IPC is only found in insect stage procyclic parasites (13). This species proved to be susceptible to sphingomyelinase (which specifically breaks down SM into phosphorylcholine and ceramide), thereby confirming its identity (figure 3B). These data are in support of previous mass spectrometry analyses which showed that the parasite harbours both SM and IPC phosphosphingolipids (13) and suggest that TbSLS is a novel bi-functional enzyme acting as both an SM and an IPC synthase.

#### *In vitro* analyses of TbSLS1 activity

To further investigate the function of TbSLS, microsomal material isolated from the TbSLS1 complemented yeast was used in an *in vitro* assay. As expected from the metabolic labelling studies, the addition of NBD C<sub>6</sub>-ceramide to this lipid fraction lead, under the conditions employed, to the synthesis of two distinct products – one

comigrating with IPC; the other with SM (data not shown). In order to delineate enzyme function this *in vitro* assay was refined according to data obtained from the analysis of LmIPCS (Mina *et al.* in preparation). In brief, the microsomal fraction contaminated with endogenous (i.e. yeast) substrates (PI, PC, ceramide etc.) was washed with ice-cold 2.5% 3-[3-(cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS). This facilitated analysis of the effect of adding exogenous substrate to the reaction – in this case candidate donor substrates PI, PC and phosphatidylethanolamine (PE); plus the known acceptor substrate NBD C<sub>6</sub>-ceramide. Without the addition of donor substrate the CHAPS-washed membranes demonstrated a relatively low level of IPC/SM synthase activity. The addition of bovine PI led to a large (more than 12-fold) increase in the formation of IPC demonstrating that TbSLS1 functions as an IPC synthase (figure 4A). In contrast bovine PC and PE - donor substrates for SM and ethanolamine phosphorylceramide (EPC) synthesis respectively - had no significant effect on enzyme activity (figure 4A and B). Notably, similarly treated *S. cerevisiae* microsomes did not respond to the introduction of bovine PI indicating that the yeast IPC synthase is substrate specific (figure 4C). Given the sphingolipid profile revealed by NBD C<sub>6</sub>-ceramide labelling of complemented yeast cells and microsomes, the lack of apparent SM synthase activity following the addition of PC was surprising (SM synthesis; figure 4). However, when PI and PC were added simultaneously the quantity of IPC produced decreased 5-fold indicating that the 2 potential donor substrates compete to bind to the same region of the enzyme, this affect was not seen in the ScaUR1 control (figure 4A and C).

Taken together these results indicate that TbSLS1 functions as an IPC synthase but also binds PC and participates in the synthesis of SM. The lack of *in vitro* SM synthase activity in the presence of exogenous PC may relate to substrate specificity (see

ScAUR1 and PI, figure 4C), with the enzyme being unable to utilize bovine liver PC (a mixed natural product with predominantly C36:2 PC; Avanti Polar Lipids) employed in the assay. In contrast, *S. cerevisiae*, where both TbSLS1 and 4 appear to function as SM synthases, possesses predominantly C32:2 and C34:2 PC (3). Perhaps TbSLS favours these relatively short acyl groups? However, *T. b. brucei* procyclic and bloodstream forms harbour significant quantities of C36, C38 and C40 PC species (23, 26, 27), indicating that any substrate selectivity is due something more subtle. In support of this, although TbSLS1 can utilize bovine liver PI (predominantly C38:4) efficiently as a substrate for IPC synthesis, procyclic form parasites (known to synthesize IPC (13)) harbour only trace levels of the C38:4 donor substrate.

#### *Inhibition of TbSLS using a known anti-fungal agent*

IPC synthase is a recognized target for anti-fungal drugs and the natural product aureobasidin A is widely utilized and specific experimental inhibitor (29). This drug also specifically inhibits the activity of *Leishmania* LmIPCS, a TbSLS orthologue (7; Mina *et al.*, in preparation) but at levels several orders of magnitude higher than those with the *S. cerevisiae* enzyme.

Using TbSLS1 complemented yeast in an agar diffusion assay it is clear that the enzyme is sensitive to aureobasidin A at concentrations considerably less than 25  $\mu\text{M}$ , and as such is less refractory to the drug than LmIPCS and similar to the yeast enzyme in this system (7). Cycloheximide (which inhibits protein translation) and myriocin (which inhibits the enzyme involved in the first step of sphingolipid synthesis, serine palmitoyltransferase) were used in the assay as positive controls (figure 5A).

To establish the  $\text{IC}_{50}$  value of aureobasidin A against the *T. b. brucei* enzyme the *in vitro* assay was employed using CHAPS-washed membranes with NBD C<sub>6</sub>-ceramide

and PI as receptor and donor substrates respectively. The synthesis of labelled IPC was used as a measure of IPC synthase activity. From this assay it was evident that the enzyme is acutely sensitive to the drug with the activity calculated to be 50% inhibited ( $IC_{50}$ ) by approximately 0.2 nM aureobasidin A (figure 5B). This is comparable with the value for the *S. cerevisiae* enzyme (21).

#### *Validation of TbSLS as a target of anti-protozoals*

Using a sequence fragment common to all four TbSLS isoforms an RNAi construct was prepared in the p2T7 vector (17), and used to specifically inhibit their expression in cultured bloodstream form *T. brucei* (SMB). Non-induced TbSLS RNAi cells grew in a similar manner to control SMB parasites carrying empty vector with or without the doxycycline. In contrast, doxycycline induction of TbSLS RNAi saw the parasites cease division and led to some cell death as scored by light microscopy. RT-PCR, using  $\beta$  tubulin as a control, confirmed the specificity of the TbSLS mRNA inhibition (figure 6).

This genetic approach validated TbSLS as an essential enzyme for pathogenic bloodstream form parasite growth, and so a potential drug target. Given the *in vivo* and *ex vivo* data shown above in which aureobasidin A was demonstrated to inhibit TbSLS1, the efficacy of this natural compound was tested against cultured bloodstream form *T. b. brucei* (Lister 427; figure 7). When the concentration of aureobasidin A was 1  $\mu$ M cell growth was completely inhibited and the parasites were scored as dead by light microscopy within 24 hours. The  $EC_{50}$  of aureobasidin A against the parasites was estimated from these data as being below 250 nM. However, it is the typanocidal activity of this compound at higher concentrations that is of most significance.

## **Summary**

Previous studies using pharmacological and genetic inhibition of the first step in sphingolipid biosynthesis, catalysed by serine palmitoyltransferase, showed that this pathway is essential for the viability of both bloodstream and procyclic forms of *T. b. brucei* (10, 30). This is in contrast to the related protozoan parasite *Leishmania major* where this enzyme, though essential for sphingolipid biosynthesis, is non-essential for both viability and pathogenesis (6, 34). These studies indicate that sphingolipid biosynthesis could be a viable drug target in the African trypanosomes.

The sphingolipid biosynthetic pathway is largely conserved across the Eukaryota however, whilst animal cells synthesize the phosphosphingolipid sphingomyelin (SM) it is known that yeast, plants and at least some of the protozoa produce inositol phosphorylceramide (IPC) (18). The IPC synthase of the pathogenic fungi has long been validated and studied as a novel drug target (12), and the recent identification of a functional orthologue in the protozoan Kinetoplastids, the causative agents of several so-called neglected diseases, has led to its consideration as a target for anti-protozoal agents (7). In this study we confirm that the *T. brucei* orthologue (TbSLS) of the *Leishmania* IPC synthase (LmIPCS) is also a functional orthologue of the *S. cerevisiae* enzyme encoded by AUR1. However, unlike the *Leishmania* enzyme TbSLS is able to catalyze the synthesis of both IPC and SM, which reflects the known sphingolipid content of *T. brucei* cells (13). Using an *in vitro* assay system utilizing TbSLS1 complemented AUR mutant yeast microsomes, it was demonstrated that the *T. brucei* enzyme was able to function as an IPC synthase. However, SM synthase activity could not be constituted in the same manner perhaps due to some level of specificity for the PC donor substrate. Notably, substrate selectivity is also demonstrated by the yeast enzyme, AUR1p, with respect to PI.

The known yeast and fungal IPC synthase inhibitor, aureobasidin A, has previously been shown to be active against the related kinetoplast, *Leishmania* species, inhibiting growth, but not affecting viability, in culture (32). However, it has been demonstrated that the *L. major* IPC synthase is refractory to aureobasidin A (7) and that its effect against the parasite in culture is non-specific (6). A similar situation has been observed with respect to the causative agent of Chagas disease, *T. cruzi* (8). In contrast, this study showed that aureobasidin A is specifically active against TbSLS1 complemented auxotrophic AUR1 yeast mutant. Furthermore, the drug demonstrated a high level of efficacy against the parasite enzyme activity *in vitro*, with an IC<sub>50</sub> of approximately 0.2 nM. In light of these results demonstrating the ability of a specific inhibitor to affect enzyme activity, it was important to validate TbSLS as a potential target of anti-HAT drugs. Simultaneous RNAi of all four closely related isoforms of the enzyme demonstrated that the activity is essential for growth and viability and so represents a new, much needed anti-protozoal target. Furthermore, aureobasidin A proved highly effective and trypanocidal against cultured bloodstream form *T. b. brucei*, with a submicromolar EC<sub>50</sub>.

Together these data raise the possibility of the discovery of a new generation of lead inhibitors directed against TbSLS ultimately leading to novel drugs for the treatment of Human African Sleeping Sickness.

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## Figure Legends

### Figure 1

The predicted amino acid sequences of the 4 previously identified *T. b. brucei* orthologues (TbSLS1-4; Tb09211.1000, Tb09211.1010, Tb09211.1020 and Tb09211.1030) of the *Leishmania* IPC synthase are highly related demonstrating in excess of 90% identity. Illustrated in a ClustalW alignment. TM, TbSLS1 predicted transmembrane domains (PHD Protein Predict); D, domains conserved with respect to SM synthase and *Leishmania* IPC synthase; \* conserved residues; : highly similar residues; . similar residues; \* residues forming catalytic triad.

### Figure 2

Transformation with pRS426 TbSLS1 rescues the auxotrophic mutant YPH499-HISGAL- AUR1, as does pRS424 AUR1. YPH499 (wild type) does not grow on minimal media in the absence of histidine (HIS) and uracil (URA); YPH499-HIS-GAL-AUR1 pRS426 (empty vector) does not grow in the presence of glucose.

Figure 3

A. Metabolic labelling of YPH499-HIS-GAL-AUR1 TbSLS1 and TbSLS4 complemented yeast with NBD C<sub>6</sub>-ceramide showed that they are able to synthesize 2 identifiable sphingolipid species. One co-migrated with IPC the other with SM.

B. Sphingomyelinase (SMase) treatment of the same extract demonstrated that the predominant labelled species in TbSLS1 complemented YPH499-HIS-GAL-AUR1 contains SM. Normalized with respect to control.

NBD C<sub>6</sub>-ceramide labelled yeast lipid extracts fractionated by HPTLC. O, origin; IPC, inositol phosphorylceramide; SM, sphingomyelin; Cer, ceramide (migrating at the front); SMase, sphingomyelinase.

#### Figure 4

Detergent-washed microsome extracts from YPH499-HIS-GAL-AUR1 TbSLS1 yeast demonstrate IPC synthase activity on the addition of PI, but not SM activity on the addition of PC. NBD C<sub>6</sub>-ceramide labelled products fractionated and quantified following reaction as described. All results normalized with respect to control.

A. The addition of bovine liver PI constitutes maximal IPC synthase activity from TbSLS1, leading to a greater than 12-fold increase in IPC over (Control). This activity is 5-fold suppressed in the presence of an equimolar quantity of bovine PC.

B. The addition of PC (nor PI or PE) did not lead to an increase in SM, indicating that it is not functioning as a substrate.

C. Yeast IPC synthase activity is not induced by the addition of bovine liver PI indicating that whilst it is a substrate for TbSLS1 it is not for ScaUR1.

IPC, inositol phosphorylceramide; SM, sphingomyelin; Control, no donor substrate added; PI, bovine liver phosphatidylinositol; PC, bovine liver phosphatidylcholine; PE, bovine liver phosphatidylethanolamine.



## Figure 5

The fungal IPC synthase inhibitor is active against TbSLS activity.

A. Agar diffusion assay: Volumes of 1, 2 and 3  $\mu$ l of 25 and 100  $\mu$ M Aureobasidin A (AbA), 1mM myriocin (MYR), 25 $\mu$ M cycloheximide (CYC) and DMSO as a control spotted onto YPH499-HIS-GAL-AUR1 TbSLS1 plates.

B. TbSLS1 IPC synthase activity determined *in vitro* in the presence of aureobasidin A (AbA). Activity is scored as 100% in the absence of the inhibitor. The IC<sub>50</sub> was calculated to be approximately 0.2 nM.

## Figure 6

TbSLS is an essential enzyme in bloodstream form *T. brucei*.

A. Inhibition RNA of TbSLS. Cell counts over 48 hour period:  $\blacktriangle$  Mock transfected cells non-induced;  $\times$  Mock transfected cells induced by 1  $\mu$ g/ml doxycycline ;  $\blacklozenge$  TbSLS RNAi cells non-induced;  $\blacksquare$  TbSLS RNAi cells induced by 1  $\mu$ g/ml doxycycline. Error bars for standard deviation over three replicates are shown.

B. RT PCR using total RNA isolated from TbSLS RNAi parasites with or without doxycycline induction (Deoxy). RT, reverse transcriptase.  $\beta$ TUB,  $\beta$  tubulin control.

## Figure 7

Aureobasidin A is trypanocidal against bloodstream form *T. b. brucei*. Cell counts over 72 hours with:  $\blacklozenge$  1  $\mu$ M aureobasidin A (AbA);  $\blacksquare$  250 nM AbA;  $\blacktriangle$  control. Error bars for standard deviation over three replicates are shown.



Figure 2

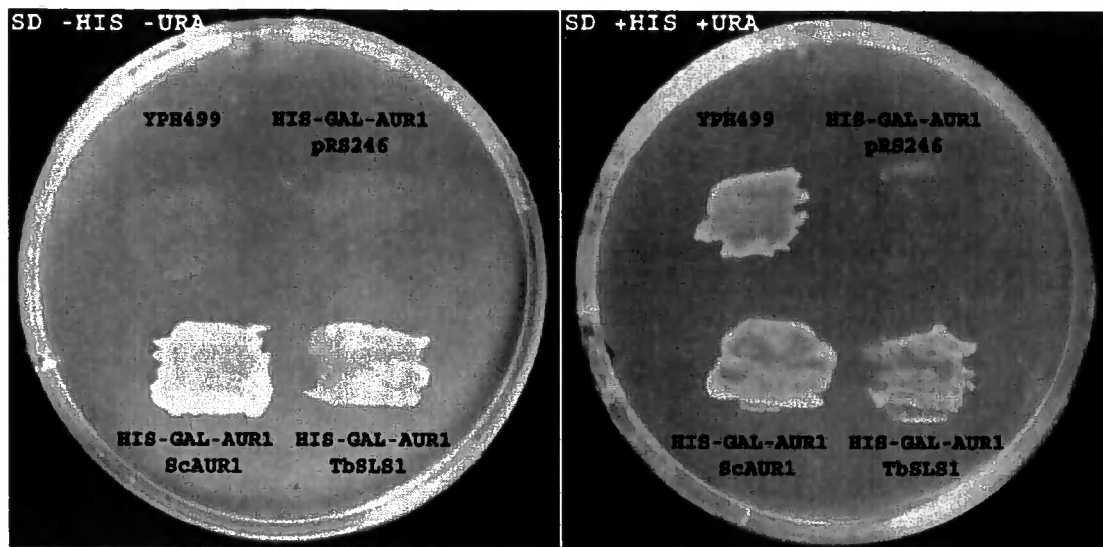
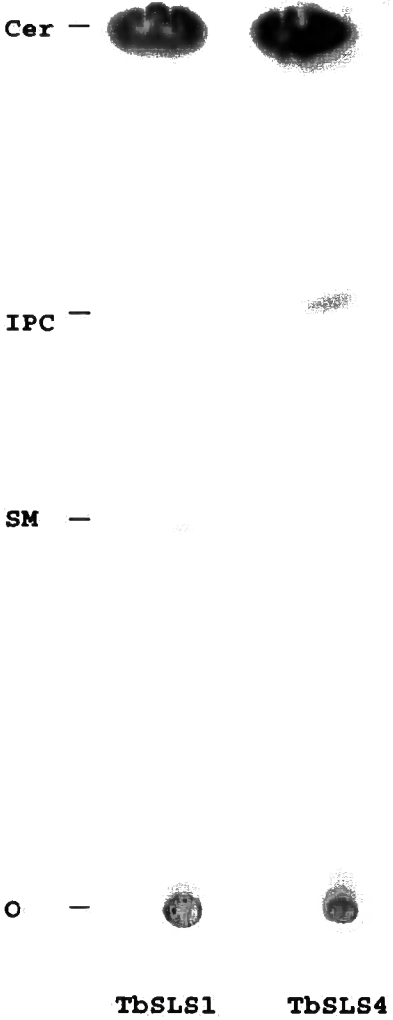


Figure 3

A



B

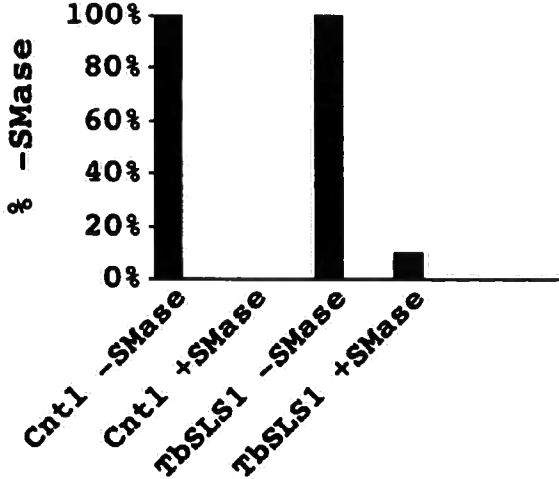


Figure 4

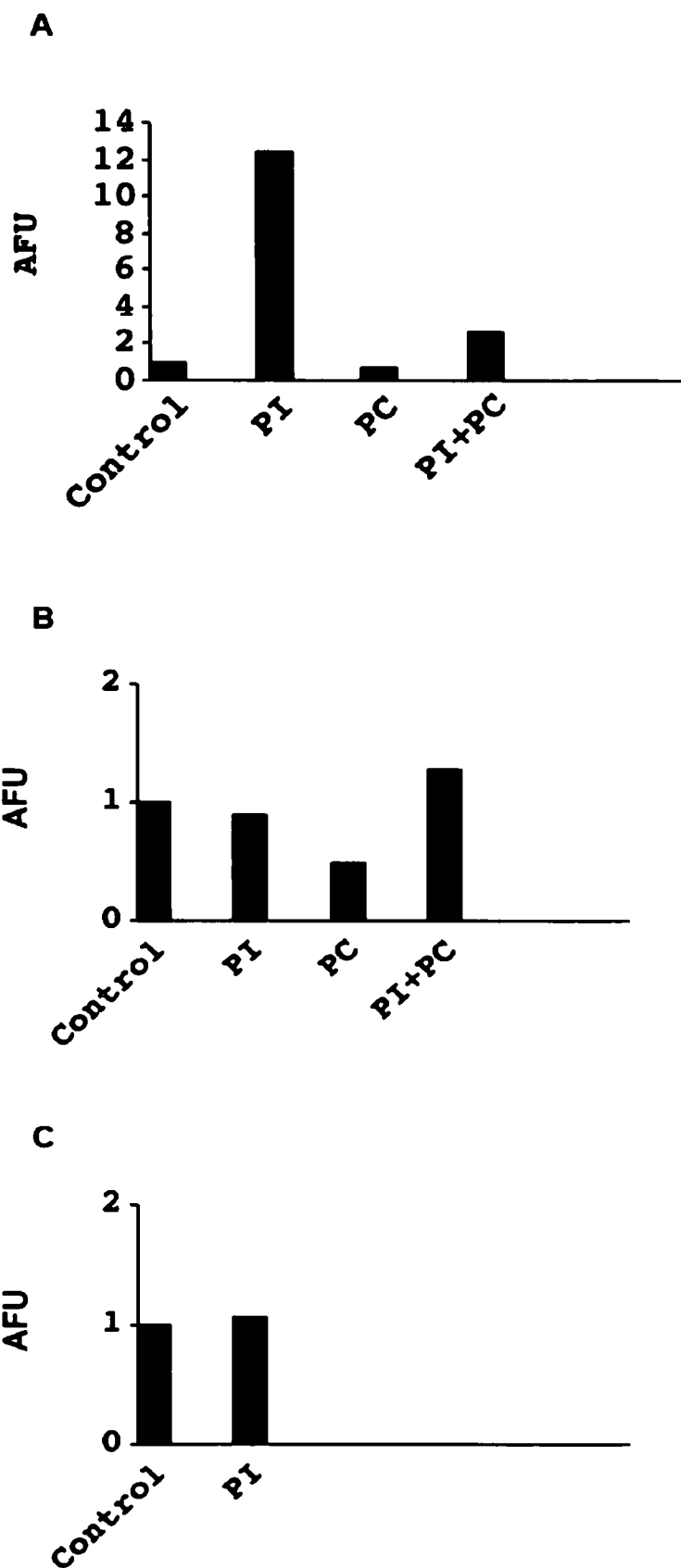
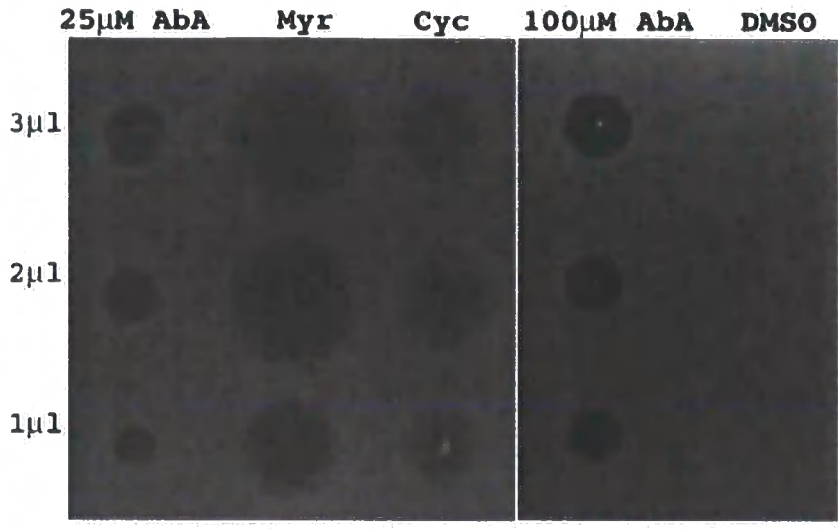
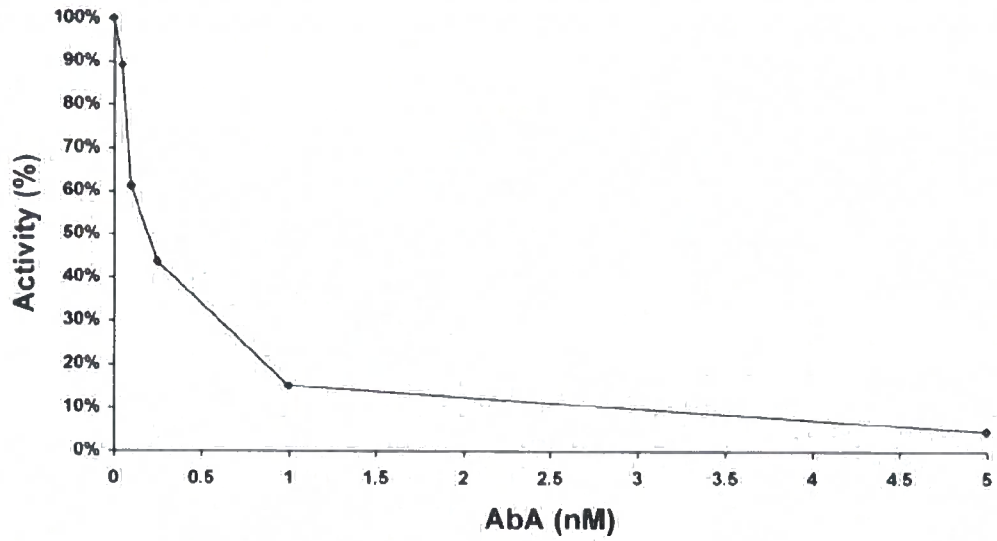


Figure 5

A

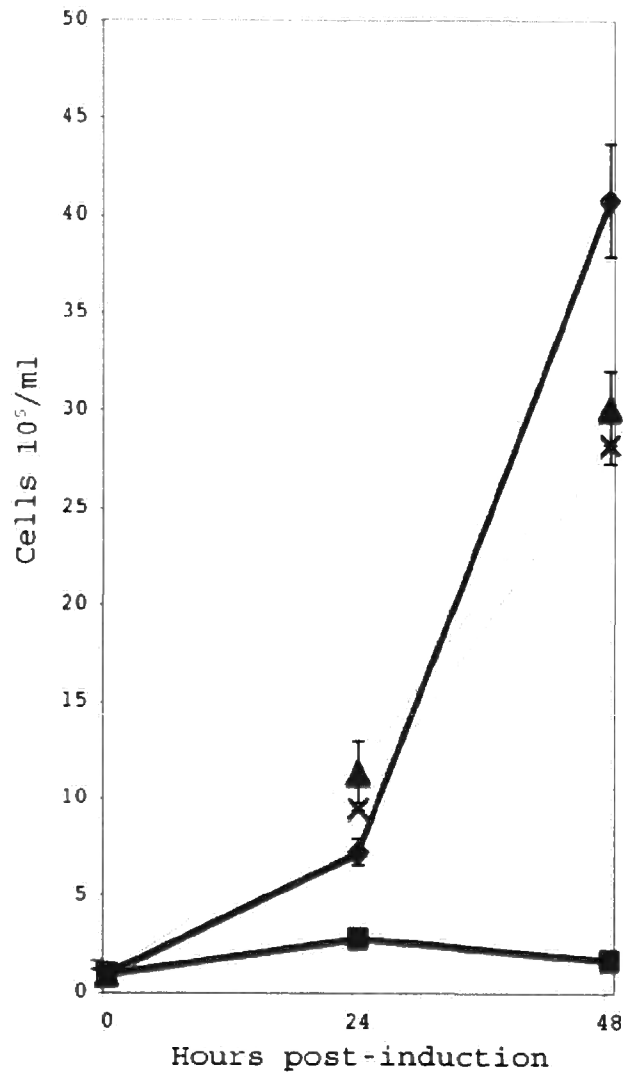


B



**Figure 6**

**A**



**B**

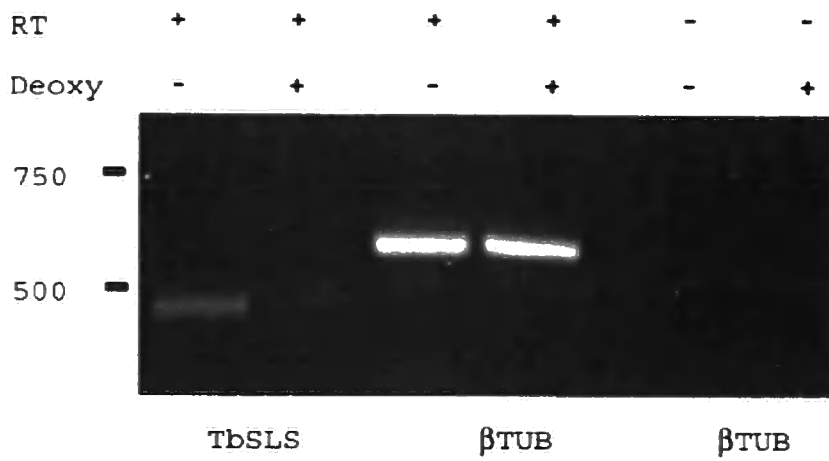


Figure 7

