Diagnostics and Chemotherapy of *Trypanosoma brucei*

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PhD Thesis

Submitted by

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CONTENTS

Abstract

Abstract

Trypanosoma brucei is a tsetse fly-transmitted protozaon pathogen of the genus *Trypanozoon* that causes sleeping sickness or Human African Trypanosomiasis (HAT) in man and nagana in animals. HAT is invariably fatal unless treated and causes huge economic losses to the affected communities. Therefore HAT management is of great importance in improving the livelihoods of such communities. HAT management involves accurate diagnosis and chemotherapy. However, HAT is a neglected disease with respect to treatment as well as diagnostics. In this thesis I set out to address some of the problems related to HAT diagnostics and chemotherapy. This work comprises five (5) chapters. Chapter 1.0 which is the Introduction: gives a general overview on *Trypanosoma brucei* and HAT. Chapters 2.0, 3.0 and 4.0, comprise the results. Chapter 2.0 is an aspect of HAT diagnostics while chapters 3.0 and 4.0 are aspects of chemotherapy. Chapter 2.0 is Comparative genomics and Serodiagnostic evaluation of Tandem Repeat (TR) Proteins in *Trypanosoma brucei.* In the absence of laboratory equipement, serology is the main stay for diagnosis of HAT under field conditions. *Trypanosoma brucei* TR proteins were retrieved and identified *in silico* and genes of selected proteins with expression profiles in the bloodstream form of the parasite were amplified and expressed in *E. coli.* The recombinant proteins were purified and evaluated using ELISA. Preliminary results obtained show that the selected proteins cannot be used as antigens for HAT diagnosis since they do not distinguish between sera from HAT patients and that from healthy individuals. Chapter 3.0: Mechanisms of drug resistance in *Trypanosoma brucei* is divided into three parts i) Combined contribution of TbAT1 and TbMRPA to drug resistance in *Trypanosoma brucei.* TbAT1 is an aminopurine transporter responsible for uptake of trypanocides like melarnophenyl arsenicals, pentamidine, diminazene aceturate, isometamidium, cordycepin and tubercidin, while TbMRPA is an efflux transporter of the ABC type which contributes to melanophenyl arsenical resistance

when overexpressed. The aim of this work was to investigate what happens when both loss of TbAT1 and gain in TbMRPA, coincide in the same cell. Results obtained show that the two transporters independently contribute to arsenical resistance in *Trypanosoma brucei* (Chapter 3.0a). Part ii (Chapter 3.0b) and iii (Chapter 3.0c), investigate the phenomenon of cross-resistance between melanophenyl arsenicals and diamidines in *Trypanosoma brucei*. Drug resistance to melarsoprol and pentamidine was independently induced in the laboratory strains and the mechanisms involved were investigated. In chapter 3.0b the mechanism for cross-resistance is not known since only the melarsoprol-resistant isolate had lost the TbAT1. In chapter 3.0c however, cross-resistance is associated with the loss of the High Affinity Pentamidine Transporter (HAPT1) activity. In Chapter 4.0: Comparative genomics of metabolic networks from parasites and host, the aim was to determine how networks shrink and to identify potential drug targets. In this work pathways constituting core metabolism of different organisms were broken down into individual reactions and analysed in the perspective of entire networks. Different properties of the networks were compared between parasites and non-parasites. Two enzymes specific to *Trypanosoma brucei*; methionine synthase (EC2.1.1.14) and homocysteine methyltransferase (EC2.1.1.10) were knocked down and knocked out, respectively and the generated clones were grown in methionine-free or methionine-supplemented medium. Results obtained show that network integrity rather than scale-freeness has acted as a selective principle for evolution of parasite metabolism. Experiments on methionine synthase and homocysteine methyltransferase show that *Trypanosoma brucei* is auxotrophic for methionine and that homocysteine methyltransferase and methionine synthase are potential drug targets. Chapter 5.0 is the overall discussion and outlook of all the work done and written in this thesis.

Chapter 1.0

Introduction

Chapter 1.0
Introduction

1.1 Biology of *Trypanosoma brucei*

1.1.1 Classification

Trypanosoma brucei is a unicellular protozoan parasite of the genus *Trypanosoma* and subgenus *Trypanozoon*. *Trypanosoma* belong to the class *kinetoplastida* and order *Trypanosomatida*. Details of the classification are shown in Figure 1. The name *Trypanosoma* is derived from two Greek words *trypaö* and *soma*, which means boring and body, respectively because of their corkscrew-like motion (http://en.wikipedia.org/wiki/Trypanosoma). The trypanosomes of mammals are divided into two categories depending on the location of the parasite within the vector and hence the method of transmission. The Salivaria occupy the salivary glands or mouth parts and midgut of the tsetse fly and are transmitted during feeding. Biologically speaking, the tsetse is the finite host where sexual reproduction takes place. On the other hand the Stercoraria develop in the posterior station, usually the hind gut and the rectum. Transmission of the Stercoraria is by depositing faeces containing infective forms on the skin of the vertebrate host by the vector. *T. brucei subsp.* belong to the Salivarian category (Molyneux and Ashford, 1983).

Trypanosoma brucei consists of three morphologically indistinguishable subspecies; *Trypanosoma brucei brucei* (*T. b. brucei*) which causes nagana in cattle, and *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*) and *Trypanosoma brucei gambiense* (*T. b. gambiense*) which cause sleeping sickness or Human African Trypanosomiasis (HAT). *T. b. rhodesiense* is the causative agent of the acute form of HAT while *T. b. gambiense* is the causative agent of the chronic form. However, both forms are lethal unless treated. Nagana is a group of

diseases of ruminants, camels, equines, swine and carnivores. It is caused by tsetse - transmitted *T. congolense, T. vivax, T. simiae, T. b. brucei*

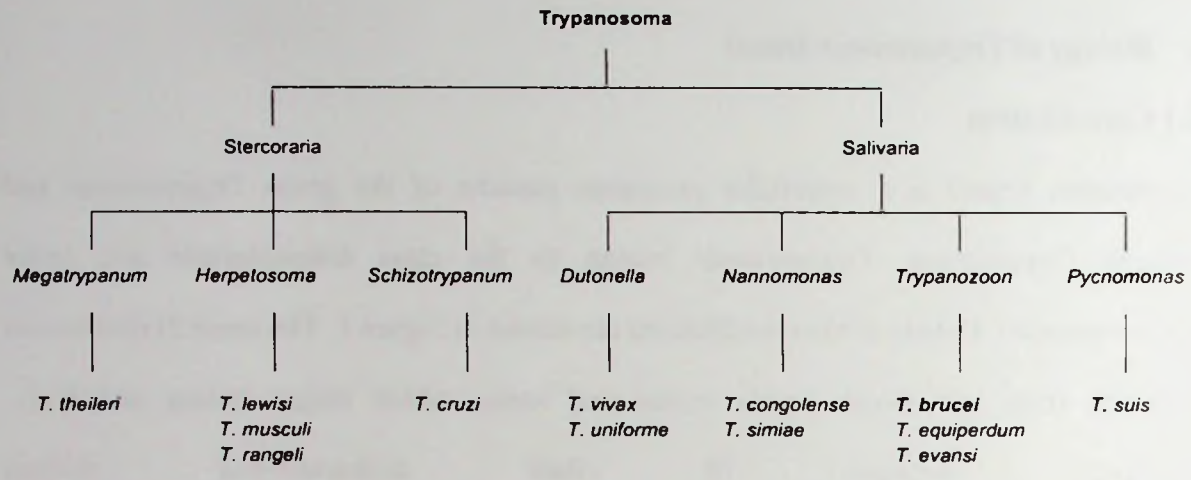

Figure 1. Phylogeny of *Trypanosoma* after Molyneux and Ashford.

and *T. suis. T. b. brucei* is virulent among horses, donkeys, camels and dogs. *T. b. brucei* also causes diseases in cattle, goats, sheep and pigs, but to a lesser virulence (Maudlin *et al*, 2004). The *T. brucei subsp.* are found in the blood and tissues of their mammalian hosts and are transmitted from one host to another by the tsetse fly. Hence the distribution of trypanosomiasis in Africa is almost absolutely determined by the distribution of the tsetse fly (Jordan, 1986, Dietmar, 2008).

T. brucei (Figure 2) is a spindle-shaped cell (20 to 30 by 1.5 to 3.5μm) with a single flagellum which emerges from the posterior end and runs along the cell membrane and extends beyond the anterior part of the cell. The flagellum is attached to the cell membrane by an undulating membrane. The base of the flagellum is associated with the kinetoplast which is a large particle containing the mitochondrial DNA. *T. brucei* appears in two forms; the trypomastigote form which is found in the mammalian host and the epimastigote form

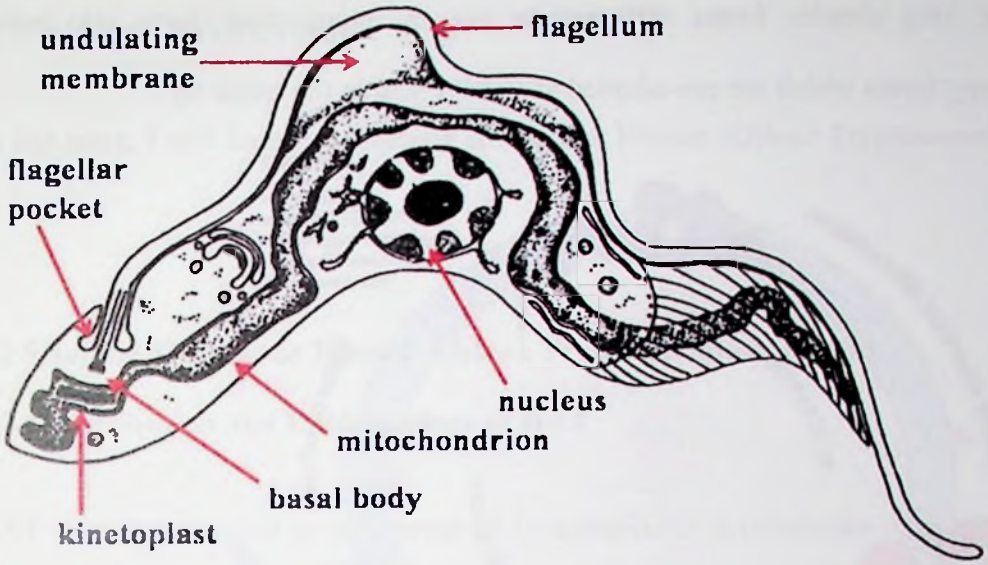

Figure 2: Structure of *Trypanosoma brucei* by K. Vickerman, 1985

which is found only in the tsetse fly. *T. brucei subsp* are exclusively extracellular (Chappuis *et al*, 2005).

1.1.2 Life Cycle of *Trypanosoma brucei*

T. brucei subsp. are digenic pathogens whose life cycle (Figure 3) completion depends upon the alteration between a vertebrate host and the invertebrate host; the tsetse fly. When a tsetse fly takes up a blood meal from an infected mammalian host, it ingests short stumpy forms of trypanosomes present in the blood or lymph. The ingested trypanosomes loose their glycoprotein surface coat (VSG). In the midgut of the fly, they transform into procyclic trypomastigotes, multiply by binary fission, leave the midgut and differentiate into epimastigotes. The epimastigotes migrate forward toward the salivary glands where they continue multiplying by binary fission and then epimastigotes differentiate into metacyclic trypomastigotes which are pre-adapted for life in a mammalian host. Upon a bite by a trypanosome-infected tsetse fly, the metacyclic forms establish in the skin, differentiate to bloodstream trypomastigotes (long slender forms), enter the lymphatic system and pass into the bloodstream of the mammalian host. In the blood and lymphatic system the bloodstream

trypomastigotes divide by binary fission and later invade the central nervous system (CNS). The proliferative long slender forms differentiate via an intermediate form into non-proliferative stumpy forms which are pre-adapted to survive within the tsetse fly.

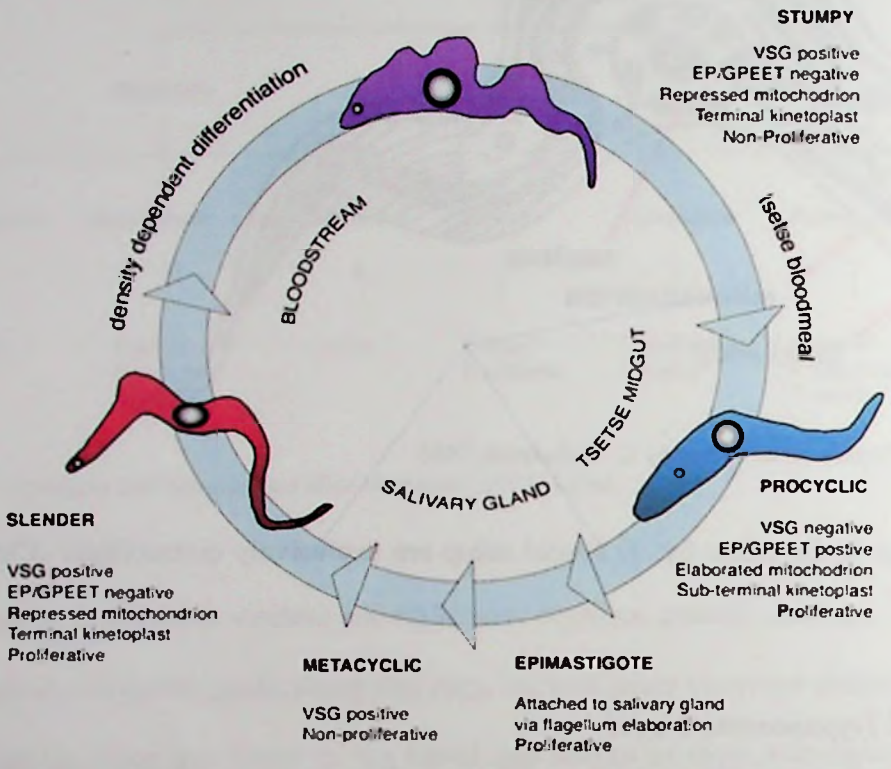

Figure 3: Life cycle of *Trypanosoma brucei.* Picture from Institute of immunology & Infection Research-Gallery of Picture, University of Edinburgh. www.biology.ed.ac.uk/research/institutes/immunology/big_image02.php) (VSG, Variant Surface Glycoprotein; EP/GPEET, procyclins).

When a tsetse fly takes a blood meal from an infected host, it is the short stumpy forms that survive within the tsetse fly (www.dpd.cdc.gov/dpdx/HTML/Frames/S-Z/Trypanosomiasis; Barret *et al*, 2007). Bloodstream and procyclic parasites are coated with variant surface glycoprotein (VSG) while procyclic parasites are coated with procyclins (EP and GPEET).

Humans are the reservoir host for *T. b. gambiense*, but this species can also be found in pigs (Paindavoine *et al*, 1986) Wild game animals are the main reservoir host of *T. b. rhodesiense*

though humans are also hosts. On the other hand domestic animals are the only known hosts of T. *b. brucei* (WHO, 1986).

In this work, I will focus on Sleeping sickness or Human African Trypanosomiasis (HAT).

1.2 Sleeping Sickness or Human African Trypanosomiasis (HAT)

1.2.1 Distribution and Epidemiology of HAT

HAT is known to occur in rural areas of 36 countries in sub-Saharan Africa at different levels of endemicity. According to current estimates, there are about 250 foci of disease transmission and 60 million people are at risk and 300,000 –500,000 are infected. The epidemiological situation in some countries is totally unknown because only a fraction of the population at risk is under appropriate surveillance (WHO, 2006). In general, *T. b. rhodesiense* HAT represents 10% of the reported cases and is a zoonosis endemic to the savanna and recently cleared bushy areas of Eastern and Southern Africa. Cattle are the main reservoir and humans are occasionally infected through an animal–fly-human cycle. Inter-human transmission is rare except during epidemics as was observed in the Busoga focus of Uganda 20 years ago (Pepin, 2007). Wild animals are usually affected but epidemics occasionally occur in domestic animals. *T. b. rhodesiense* is transmitted by the bite of *Glossina morstans* or *G. fuscipes* groups, and the human-vector contact occurs in savanna woodland but can be peridomestic during epidemics. The countries affected include Burundi, Ethiopia, Kenya, Malawi, Mozambique, Sudan, Tanzania, Southeastern Uganda, Zambia and Zimbabwe. On the other hand, *T. b. gambiense* HAT represents 90% of the reported cases and is endemic in the water holes and river areas in Western, Central and some parts of Eastern Africa (Molyneux and Ashford, 1983; WHO, 1998; Chappius *et al*, 2005; WHO, 2006). *T. b.*

gambiense is transmitted by tsetse flies of the *Glossina palpalis* or *G. fuscipes* groups (Chappius *et al*, 2005). Most active foci are Cameroon, Congo, Cote d Ivoire, Southern Sudan and northwest Uganda (Molyneux and Ashford, 1983; Kitonga, 2002). The distribution of HAT is shown in Figure 4.

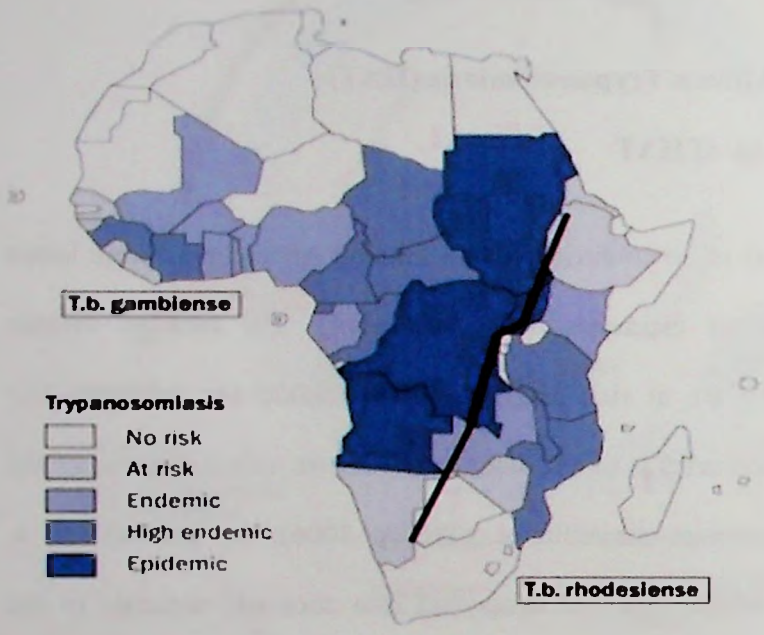

Figure 4: Distribution of Sleeping sickness in Sub-saharan Africa (WHO, www.who.int/csr/resources/publications/CSR_ISR_2000_1trvps/en/index.html). The countries shown in deep blue are currently reporting in excess of 1000 cases per year. Nearly 97% of all reported cases are caused by *T. b. gambiense* and is found in western and central Africa while *T. b. rhodesiense* is found in East and Southern Africa (Barrett *et al.* 2007).

There have been three severe epidemics of African trypanosomiasis over the last century; one between 1896 and 1906, was mostly in Uganda and the Congo Basin, another in 1920 occurred in several African countries and the third one began in 1970 and is still in progress. The 1920 epidemic was arrested using mobile teams, which systematically screened millions of individuals at risk. The disease had practically disappeared between 1960 and 1965. When screening and effective surveillance were relaxed, the disease reappeared in several foci over the last forty years. The resurgence of infection has also been facilitated by civil war, population movements, economic decline, reduced health financing, and lack of human resources in these areas (Leder and Weller, 2008; WHO, 2006).

1.2.2 Clinical manifestation of HAT

Infection starts with the bite of an infected tsetse fly (*Glossina* spp.) and signs and symptoms are classified according to the clinical progression of the disease as described below.

1.2.2.1 The Initial Trypanosomal Chancre

A few days after the bite of an infected tsetse fly, the chancre, a tender, painful swelling is frequently observed in *T. b. rhodesiense* infections, but is less common with *T. b. gambiense*. Trypanosomes are present in the inflammatory tissues and this primary lesion can last up to several weeks.

1.2.2.2 The Haematolymphatic Or First Stage

The onset is variable and episodes of fever lasting 1-7 days occur together with generalized lymphadenopathy. Fever results from entry of the parasites into the blood and their production of pharmacologically active substances (Keiser, 1999). The pattern of fever is irregular and is a consequence of the progressive waves of parasites multiplying in the blood. This stage is characterized by headache, weight loss, joint pains, skin rashes, pruritus, anemia or endocrine disorders. Sometimes the symptoms are minor and may not alert the patients to seek medical advice. In *T. b. gambiense* infections, lymphadenopathy in the posterior triangle of the neck (Winterbottom's sign) is characteristic (Keiser, 1999; Stich *et al*, 2002; Sternberg 2004; Vincedeau and Bouteille, 2006). In *T. b. rhodesiense* infections, symptoms at this stage can already be severe and without treatment, one tenth of the patients will die due to myocardial involvement. In both forms of the disease hepatosplenomegaly and a faint rash are other non-specific signs. (Stich *et al,* 2002).

1.2.2.3 The meningoencephalitic or second stage

The second stage occurs when parasites cross the blood-brain barrier by unknown mechanisms and invade the central nervous system (CNS). In *T. b. rhodesiense* infections, this occurs after weeks while in *T. b. gambiense* infections it occurs after months or years (Keiser, 1999, Stich *et al*, 2002).

The clinical classification of the second stage of the disease is mainly limited to measurements of changes in the cerebrospinal fluid (CSF), and clinical signs vary between individuals and disease foci (WHO, 1998). The onset of this stage is insidious and it results in a chronic encephalopathy associated with headache and mental changes. Symptoms include abnormal body movements, extrapyramidal signs, tremors, irritability, and alterations in mood, indifference or the classical reversal of the sleep rhythm with day time somnolence and night insomnia. As the disease progresses, the neurological abnormalities become more marked: Epileptic seizures, local paralyses, maniacal, total indifference and somnolence leading to coma and death may occur. Due to the long duration of *T. b. gambiense* infections, extensive neurological changes might be observed, which may lead to acute infections or malnutrition and subsequent death (Stich *et al*, 2002, Sternberg 2004; Vincedeau and Bouteille, 2006).

1.2.3 Immunology of HAT

The inoculation of trypanosomes in a mammalian host triggers a series of event which involve first the innate immunity and secondarily specific immunity responses, both of which fail to eliminate the parasites.

1.2.3.1 Natural Immunity

Normal human sera contain apolipoprotein L-1 (APOL1); a trypanolytic factor which is responsible for resistance to *T. b. brucei, T. congolense* and *T.evansi* infections. APOL1 is a human-specific apolipoprotein bound to high-density lipoproteins and is taken up in the parasite by endocytosis. In the parasite, APOL1 triggers the formation of anion-selective pores in the lysosomal membrane thereby inducing uncontrolled osmotic swelling of the compartment and subsequent cell death. Lack of the APOL1 depletes the serum of trypanolytic activity. However, *T. b. rhodesiense* and *T. b. gambiense, subsp.* have acquired resistance to APOL1. *T. b. rhodesiense* has a lysosomal protein; serum resistance-associated protein (SRA) a truncated form of VSG which interacts strongly and specifically with APOL1 in the parasites lysozome. The mechanism of resistance of *T. b. gambiense* to APOL1 is not known (Vanhollebeke *et al*, 2006, Etienne *et al*, 2006).

1.2.3.2 The Chancre

The chancre is a local skin reaction that is induced by trypanosome proliferation and it occurs a few days after trypanosome inoculation. This corresponds to the first response by the host and is common in *T. b. rhodesiense* infections. In efferent lymphatic vessels, trypanosomes are detected in the lymph 1-2 days before the chancre and their number declines during the development of the chancre and later increases again.

1.2.3.3 Antigenic variation and Antibody responses

Antigenic variation is the primary immune–evasion strategy by African trypanosomes. Trypanosomes in the mammalian host are covered with a coat of 10^8 variant surface glycoprotein (VSG) molecules attached to the cell membrane via a glycosylphosphatidyl-inositol (GPI) anchor (Magez *et al*, 2002). VSG enables trypanosomes to avoid the host

specific immune response by sequentially expressing antigenically distinct VSG or Variant Antigen Type (VAT). This is in agreement with findings that variable antigen types (VATs) are observed during infection. Remission in parasitaemia is associated with the production of antibody specific to the VAT that is predominant at the peak of the parasitaemia. However, not all trypanosomes are cleared by the antibody due to the presence of a small number of heterotypes carrying a new surface coat (new VAT) which survive the antibody produced against the antigens in the previous parasitaemic waves. The trypanosomes bearing the new surface coat continue to multiply resulting in rising parasitaemia until antibody specific to the new antigen type is produced and the cycle is repeated (Molyneux and Ashford, 1983). The parasite has a repertoire of more than 1000 transcriptionally inactive VSG genes and a single active transcription site. As a result, there is a continuous switching of VSG genes at a rate of between 10^{-3} and 10^{-4} per cell division and enables the parasite to maintain a state of chronic infection in the host (Sternberg, 2005). The VSG is the immunodominant antigen and it constitutes an important molecular interface between trypanosomes and the host. The VSG is capable of inducing both the T cell-dependent and -independent B-cell responses, depending on its conformation (Mansfield, 1994). VSG prevents trypanosome lysis by complement alternative pathway and elicits polyclonal B-cell activation which in the human infection results in generation of autoantibodies and immune complexes (Vincendeau and Bouteille, 2006).

The main feature in African trypanosomiasis is a dramatic increase in immunoglobulin (Ig) levels especially the IgM. These include both trypanosome-specific antibodies and non-specific antibodies produced when B cells are activated by cytokines. Some of the antibodies are raised against autoantigens as a result of non-specific polyclonal activation of B cells producing natural antibodies. Other autoantibodies are antigen-driven antibodies induced by

molecular mimicry (Vincendeau and Bouteille, 2006). Autoantibodies detected during African trypanosomiasis are directed against red blood cells, nucleic acids (DNA and RNA), (Kobayakawa *et al*, 1979), liver and cardiolipids (MacKenzie and Boreham, 1974), intermediate filaments (Anthoons *et al*, 1986), rheumatoid factors (Kazyumba *et al*, 1986), and components of the CNS myelin: gylcophospholipids and galactocereborsides (Jauberteau *et al*, 1991). Other autoantibodies against not yet characterized proteins have been described in HAT patients (Asoganyi et al, 1989).

Antibodies specific to trypanosomes are induced by variant and invariant VSG epitopes, membrane, cytoplasmic and nuclear antigens, through T cell-dependent and independent pathways (Reinitz and Mansfield, 1990).

Parasites are eliminated by VSG-specific IgM which appear at high levels 3-4 days after infection. VSG-specific IgG do not seem to be involved in the destruction of trypanosomes because they appear after the disappearance of the VAT population. VAT-specific antibodies decrease to low levels while the IgM class specific to invariant epitopes remains at high levels (Vincendeau and Bouteille, 2006).

In the second stage of the disease, both trypanosome-specific IgG and IgM, are detected in CSF (Lejon *et al*, 1998). These may be due to modified plasma cells known as Mott or morular cells in the white matter and also plasma cells that form perivascular infiltrates in the brain (Kennedy, 2004).

1.2.3.4 Complement

Complement activation by both the classical and the alternative, pathways, is detected in African trypanosomiasis (Ferrante and Allison, 1983). The classical pathway is mediated by antigen-specific antibodies or immune complexes (Specific immune response) while the

alternative pathway is activated by spontaneous hydrolysis of C3 antigens in absence of antibodies (non-specific immune response). In both pathways, a C3 convertase cleaves and activates component C3 causing a cascade of further cleavage and activation, of other components. The end result is the formation of the membrane attack complex (MAC) which is the cytolytic end product of the complement cascade. MAC causes osmotic lysis of the target cell (Celik *et al*, 2001; http://en.wikipedia.org/wiki/Complement_(biology). Trypanosome lysis by the alternative pathways occurs only with the procyclic trypanosomes (Ferrante and Allison, 1983). In *T. b. gambiense*, the alternative pathway was incompletely activated without generation of the terminal complex which induces membrane lysis. The classical pathway against trypanosomes was also described and could be involved in parasite clearance by antibody-mediated lysis and/or opsonisation. Coated trypanosomes are lysed by antibodies with activation of complement by the classical pathway. However, during the complement activations, the appearance of soluble fragments like the C3a and C5a anaphylatoxins and the C567 complex, could on the one hand induce the chemotactism of neutrophils and monocytes and on the other hand induce the release of amines involved in vasoconstriction and an increase in vascular permeability participating in the initial inflammatory response in the chancre. These immune complexes are also involved in some adverse effects especially in tissue damage due to immune complex deposits, like thrombosis and glomerular involvement (Vincendeau and Bouteille, 2006).

1.2.3.5 Cellular immune activation

Most information available on the cellular response to trypanosomiasis has been obtained from studies done in experimental mouse models.

1.2.3.5.1 T cell responses

Manipulation of T-cell activities is centrally involved in immunosuppression with the different T-cell subsets secreting a range of cytokines like IFN-γ, TGB-β, IL-2, IL-4, IL-6, IL-10 and IL-12. *T. b. brucei* can utilize some host substances like IFN-γ and epidermal growth factor, to promote its own growth. *T. b. brucei* does this by releasing trypanosome-derived lymphocyte-triggering factor (TLTF) which triggers the production of IFN-γ by CD8+ T cells (cytotoxic T cells) (Bakhiet *et al*, 1993, Hamadien *et al*, 1999). TLTF also induces the release of TGB-β; an immunosuppressive cytokine. As a result, the T cell proliferative response to trypanosomal and other heterologous antigens, is severely attenuated; a situation caused in part by macrophage-derived substances like prostaglandin and nitric oxide (NO). Depression of IL-2 production by macrophages also contributes to the reduced T cell response, which in turn correlates to the high levels of IL-1. Other immune changes associated with progression to the chronic disease state, include the tendency towards expression of Th2 subset of CD4+ T cells which produce cytokines like IL-4, IL-5, IL-6, IL-10 and IL-13. Although IL-4 influences immunoglobulin synthesis which may be involved in controlling parasitaemia, it may be toxic in excess. On the other hand, IL-10 may be deleterious if the levels are high enough to down regulate TNF-α and hence its trypanostatic effects (Sharafeldin *et al*, 2000; Pentreath and Kennedy, 2004).

1.2.3.5.2 Macrophages

Mononuclear phagocytes play a key role in all the steps of the immune response in the inflammatory phase as antigen-presenting cells, in specific immunity and in synergy with antibodies and cytokines. Quantitative, biochemical and functional changes of mononuclear phagocytes are observed in trypanosomiasis. In *T. b. brucei*-infected mice, there was a marked expansion of macrophages in the liver, spleen and bone marrow. Macrophages can

also be involved in immunosuppressive and immunopathological phenomena. Macrophage activation results in the release of nitric oxide and proinflammatory mediators like TNF-α and IL-1 (Sternberg, 2005; Vincendeau and Bouteille, 2006).

In African trypanosome infection, several mechanisms have been proposed to drive macrophage activation and one of them is due to direct interaction with VSG and VSG-GPI anchor components. Soluble VSG carrying the GPI anchor glycosylinositol phosphate (GIP-sVSG) core and the residual dimyristoylglycerol glycerol (DMG) anchor moiety are potent macrophage-activating factors. The GIP moiety directly induces macrophage activation and TNF-α induction in macrophages after IFN-β stimulation. On the other hand, the DMG component is involved in macrophage priming (Sternberg, 2005).

Macrophages in *T. b. brucei* infected mice are able to synthesize reactive oxygen species (ROS) after triggering by phorbol myristate acetate. Trypanosomes are highly sensitive to oxygen-derived species especially hydrogen peroxide and hypochlorus acid, synthesized during phagocytosis.

Macrophages from trypanosome-infected mice also synthesize reactive nitrogen intermediates (RNI) and trypanosomes are highly sensitive to their cytostatic/cytotoxic effects.

Macrophages also secret prostaglandins (PGs) which modulate lymphocyte and macrophage functions. During *T. b. brucei* infection, the PGE2/PGF1a is reversed, with increased release of PGE2 and slow synthesis of PGF1, due to inhibition of the prostacyclin synthetase by the increased formation of oxygen radicals by the infected cells (Fierer *et al*, 1984).

Classical and alternative states of macrophage activation are observed in trypanosomiasis. In trypanosome-infected mice, induction of alternative macrophage activation results in

induction of host arginase which both decreases trypanocidal nitrosylated compound synthesis and increase L-ornithine production. The latter is important in the polyamine synthesis which is essential for parasite growth and trypanothione synthesis. (Sternberg *et al*, 2004).

1.2.3.5.3 Natural Killer cells

Natural killer (NK) cells belong to the lymphocyte lineage and have functions both in innate and acquired immune responses. NK cells kill extracellular parasites. NK cells secret cytokines especially, IFN-γ and TNF-α which play major roles in trypanosomiasis, and are in turn regulated by cytokines which can activate or inhibit NK cell functions. NK cells are also involved in the initiation of inflammatory responses through synthesis of chemokines (Vincendeau and Bouteille, 2006).

1.2.4 Pathogenesis of HAT

The mechanisms of pathogenicity are only poorly understood. Most theories suggest that immunopathological and biochemical processes cause the pathology. High parasitaemia results in an exposure of the host to high levels of toxic metabolites, lytic enzymes and immunosuppressive membrane components, which induce unregulated lymphocyte proliferation and destructive inflammatory responses (WHO, 1998).

The pathogenesis is ultimately linked to the inability of the untreated host to eliminate the parasite, since trypanosomes escape the immune system by varying their surface glycoprotein coat (Molyneux and Ashford, 1983). These variant surface glycoproteins (VSGs) are strongly immunogenic and elicit high levels of IgM antibodies which have been suggested to increase osmotic pressure, erythrocyte sedimentation rate and blood viscosity. Circulating immune complexes activate the kallikrein, kinin, complement and blood coagulation systems, which

lead to increased vascular permeability, oedema, inflammation and tissue damage (WHO, 1998). In addition, the production of autoantibodies directed against components of the brain and heart, is often reported. The brain, heart and lung are the most severely affected organs: Dysrhythmia, heart murmurs and low blood pressure may be manifest. Pericardial effusion, myocarditis and cerebral and meningeal oedema are frequent symptoms.

The most serious and fatal consequences of trypanosomiasis occur during the second stage when the CNS is invaded by the parasites (Kennedy, 1999). First and foremost, TNF-α mediated inflammatory responses have been proposed to be necessary for the parasite penetration of the blood brain barrier endothelia (Enanga *et al*, 2002). Post-mortem and mouse histopathological studies have shown that the second stage disease is characterized by inflammatory processes (Kennedy, 1999). In mouse models, the onset of these histopathological changes coincides with the up-regulation of pro-inflammatory cytokines (Hunter and Kennedy, 1992). In patients, the inflammatory response is most pronounced in the post-treatment reactive encephalopathy (PTRE) which occurs in 5-10% of patients after treatment with arsenical drugs (Atouguia and Costa, 1999).

In the first study done to analyse cytokine in CSF of second stage HAT patients, it was shown that in both *T. b. rhodesiense* and *T. b. gambiense* patients, there was increase in counter-inflammatory cytokines like IL-10 and IL-6, which seems to be a contradiction of the observation above that there is an increase in inflammatory cytokines. Since pro-inflammatory cytokines and their resultant cellular responses are important in the final stage of second stage HAT, it is suggested that before the final stage, IL-10 down regulates the production of pro-inflammatory cytokines despite continued immune stimulation. (Sternberg, 2005).

1.2.5 Diagnosis of HAT

Diagnosis of trypanosomiasis depends on both indirect and direct methods. Indirect methods involve clinical examination of the infected mammalian hosts and serological tests. On the other hand direct methods include parasitological tests, molecular tests and *in vitro* and *in vivo* tests. Indirect methods are always followed by direct methods to confirm the presence of the parasites.

1.2.5.1 Clinical examination

Clinical examination is one of the easiest tests for trypanosomiasis. In humans, it involves checking for the presence of a chancre at the place of the tsetse fly bite in case of *T. b. rhodesiense* infections and for the swelling of the lymph nodes in the neck (Winterbottom's sign), in *T b. gambiense* infections. In *T. b. brucei* infections in animals, anemia and poor health are used as indicators of trypanosomiasis. For all indirect methods, any positive test should be confirmed by the detection of trypanosomes in the blood, lymph gland fluid or CSF (Molyneux and Ashford 1983; Connor R J, FAO, 1992; Prowse, 2005).

1.2.5.2 Serological tests

Serological tests include use of Enzyme-Linked Immunosorbent Assay (ELISA) (Voller *et al*, 1975; Van Knapen *et al*, 1977; Vervoort *et al*, 1978), Immunofluorescent antibody test (IFAT) (Bailey *et al*, 1967; Wery *et al*, 1970a,b), Card Agglutination Test for Trypanosomiasis (CATT) (Magnus *et al*, 1978) and LATEX/*T. b. gambiense* agglutination test and latex agglutination antigen test. Although all these tests use selected antigens or antibodies to detect circulating antibodies or antigens their detection systems differ.

ELISA uses an enzyme-labeled anti-immunoglobulin that is specific to anti-trypanosomal antibodies in host sera and an appropriate substrate for the enzyme. Enzyme-substrate reaction is allowed to proceed for some minutes and absorbance of the reaction mixture is determined spectrophotometrically (Voller *et al*, 1975; Van Knapen *et al*, 1977; Vervoort *et al*, 1978).

Antibody ELISA detects circulating anti-trypanosome antibodies while antigen ELISA (Nantulya 1990) detects circulating antigens. The latter was developed in an attempt to identify current trypanosome infections more accurately since the former does not distinguish between current and past infections since circulating antibodies remain in the host for some time after an infection is cured (Prowse, 2005). ELISA is highly sensitive and specific. However, it requires sophisticated equipment, well-trained personnel and a lot of time. It is therefore inapplicable in simple clinical laboratories for routine examination of trypanosomiasis suspects or in the field for epidemiological studies of the disease (Isharaza 1984).

IFAT uses an anti-human globulin that is conjugated to a fluorescent dye (e.g. Fluorescein) and examination is done under a fluorescence microscope (Bailey et al, 1967; Wery *et al*, 1970a; 1970b; Prowse, 2005). This test is highly sensitive, requires small samples of sera and antigen that can be prepared in large quantities and preserved for a long time. In surveillance surveys, dry blood samples can be carried on filter papers and later moistened with PBS over reactive zones before carrying out the test. However, the test procedure is lengthy and requires specialized laboratory facilities and hence cannot be used for on spot screening of suspects. (Isharaza, 1984).

CATT (Card Agglutination Test for Trypanosomiasis) on the other hand detects agglutination between trypanosomal antigens and the antibodies in a suspect's blood as a way of diagnosis (Magnus *et al*, 1978). The agglutination is visible with a naked eye and hence no need of sophisticated equipment. CATT is a simple, sensitive and fast method that can be used for screening purposes. However, specificity remains a problem due to cross-reactivity with antibodies related to non-pathogenic trypanosomes and antibody titres do not distinguish between past and current infections. In addition, its use is limited to *T. b. gambiense* because the Litat 1.3 genes used in production of the CATT antigen is limited to *T. b. gambiense* (Molyneux and Ashford, 1983; Agranoff *et al*, 2005, Chappuis *et al*, 2005). The LATEX/*T. b. gambiense* was developed as a field alternative to CATT. It is based on the combination of three purified surface antigens; Litat 1.3, 1.5, and 1.6. It showed higher specificity but lower or similar sensitivity in recent field studies and hence further evaluations are needed before it is recommended for routine field use (Chappuis *et al*, 2005). Latex agglutination antigen test (Leak 1998) uses latex particles linked to species-or subgenus-specific anti-trypanosome antibodies to capture relevant antigens circulating in the whole blood, plasma or serum. Since there are several combining sites on the antigens molecules, an agglutination reaction occurs. This test is simple and fast to use under field conditions (Prowse, 2005).

Antibody tests can also be used in the staging of HAT since CSF of late-stage patients contains high levels of immunoglobulins, especially IgM. Increased IgM levels in the CSF are a strong potential marker of second stage HAT (Chappuis *et al*, 2005). Staging is particularly important for HAT diagnosis due to the severe side-effects of melarsoprol. Refer to Chapter two for more information on serological tests.

1.2.5.3 Parasitological tests

The most common method of parasitological diagnosis that has been used in surveillance for many years is the palpation of cervical lymph nodes and subsequent aspiration of enlarged glands. Trypanosomes can be detected in the chancre a few days earlier than in the blood. When the chancre is punctured and the fluid is obtained, trypanosomes can be detected microscopically examined as a fresh, or fixed and giemsa-stained preparation (Chappuis *et al,* 2005). Wet smears of gland aspirates are microscopically examined for the presence of parasites. This is particularly important for diagnosis of *T. b. gambiense* infections in which the parasite is not easily found in the blood.

Another parasitological test is done by examining a wet smear of blood for the presence of live trypanosomes, or that of a giemsa-stained blood smear. The latter may be laborious since parasitaemia is usually very low and trypanosomes may not be detectable, especially in *T. b. gambiense* infections. Since analysis of a wet smear or a giemsa-stained blood smear are of low sensitivity, tests involving large quantities of blood can be used (Ashford and Molyneux, 1983). The latter tests include microhaematocrit centrifugation followed by buffy-coat examination under dark ground illumination (Woo, 1970). For the miniature anion-exchange centrifugation technique (MAECT), some blood may be applied to a small DEAE (Diethylaminoethyl) cellulose column to separate trypanosomes from blood components. After centrifugation of the eluate, trypanosomes can be detected in the pellet microscopically (Lumsden *et al*, 1979). This test is very sensitive detecting down to 100 trypanosomes/ml.

1.2.5.4 Molecular tests

Molecular diagnostic techniques used in diagnosis of trypanosomiasis include polymerase chain reaction, use of species-specific probes and Loop-Mediated Isothermal Amplification (LAMP).

The Polymerase Chain reaction (PCR) has been used to detect trypanosomes in blood and CSF of HAT suspects. Blood or CSF is used for DNA extraction after which a region of the gene that is specific to the trypanosomes is amplified by PCR using primers that flank the region of interest. PCR is very sensitive and specific, and has mainly been used in diagnosis of *T. b. gambiense* infections (Kanmogne *et al*, 1996; Penchenier *et al,* 2000; Kyambadde *et al,* 2000) where parasite numbers are generally low. PCR can also be used to detect trypanosomes in both tsetse flies and cattle. This method has an analytical sensitivity of at least the DNA of one trypanosome in a sample (Moser *et al*, 1989; Prowse, 2005). However, it requires sophisticated equipment, a lot of time and well-trained personnel and hence it cannot be used in simple clinical laboratories for screening purposes (Prowse, 2005).

Another molecular method used for diagnosis of trypanosomiasis is the use of DNA probes. DNA probes have been successfully used in characterization of various parasitic organisms including African trypanosomes (Borst *et al*, 1980; Massamba and Williams, 1984; Viscindi and Yolken, 1987). The principle here is that a single-stranded DNA fragment is labeled fluorescently or with a radioisotope, and then used to probe whole parasite DNA or whole organisms. The results are revealed with autoradiography (Prowse, 2005). Subspecies-specific DNA sequences have been developed to detect DNA polymorphism within the *T. brucei* subgroup (Paindavoine *et al*, 1986). The sensitivity and specificity of species-specific DNA probes for diagnosis of trypanosomiasis have been demonstrated under laboratory conditions

with both bloodstream and vector forms of several trypanosome species (Massamba and Williams, 1984; Kukla *et al*, 1987; Ole-Moi Yoi, 1987; Gibson *et al*, 1989) and it is also possible to use this technique to characterize trypanosome infections in animals and tsetse. However, although this technique is highly specific, its sensitivity is limited to about 100 trypanosomes/ml (Masiga *et al*, 1992).

Last but not least among the molecular methods is the recently developed Loop-Mediated Isothermal Amplification (LAMP) Method. LAMP was developed by Notomi and colleagues and it amplifies a few copies of DNA with high specificity, efficiency and rapidly, under isothermal conditions. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP while an outer primer primes strand displacement DNA synthesis releasing a single-stranded DNA. The latter serves as a template for DNA synthesis which is primed by the second inner and outer primers and produces a stem-loop DNA structure. In subsequent LAMP cycling, one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem which is twice as long. The cycling continues and at the end a total of about 109 copies of the target accumulate in less than an hour and the final products are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops which are formed by annealing of alternately inverted repeats of the targets in the same strand. The products can be analysed on agarose gels. LAMP can also be applied to RNA by using Reverse Transcriptase together with DNA polymerase (Notomi *et al*, 2000). The LAMP reaction appears to be limited by the amount of deoxynucleoside triphosphates and primers. As a result, a large amount of pyrophosphate ion is produced. The latter reacts with

magnesium ions in the reaction to form magnesium pyrophosphate as a by-product. Magnesium pyrophosphate is a white precipitate and allows easy and rapid visualization of the amplified target DNA (Kuboki *et al*, 2003). Alternatively, the synthesized DNA can be visualized with fluorescent probes like Cy3. LAMP has been used in detection of different species of trypanosomes (Kuboki *et al*, 2003; Oriel *et al*, 2007; Njiru *et al*, 2007; Njiru *et al*, 2008).

1.2.5.5 Other tests

Other tests include *in vitro* culture and animal (*in vivo*) inoculation, proteomic signature analysis and Imaging techniques. The high cost and long delay before obtaining results are obstacles to the routine field use of *in vitro* culture and animal inoculation. Proteomic signature analysis is highly sensitive and specific and though impracticable in the field it can be used to identify biomarkers that can be used to develop more conventional and simpler tests. Imaging techniques reveal nonspecific features that are useful to diagnose and stage the disease (Chappuis *et al*, 2005). Nuclear magnetic resonance (NMR) spectroscopic metabolic phenotyping of urine and plasma can be used to characterize temporal responses of patients to trypanosome infection and hence can be used for diagnosis and staging of the disease (Wang *et al*, 2008). Brain magnetic resonance imaging may show white matter abnormalities, ventricular enlargement, hyperintensities in the basal ganglia and signs of meningitis. However, these techniques are not available in endemic areas (Chappuis *et al*, 2005).

Central nervous system (CNS) involvement is detected by the presence of parasites in the cerebral spinal fluid (CSF) after lumbar puncture. If trypanosomes are not found by direct examination, then centrifugation can be done to concentrate the trypanosomes (Molyneux and Ashford, 1983) or PCR can be used (Kanmogne *et al*, 1996; Penchenier *et al,* 2000;

Kyambadde *et al*, 2000; Chappuis *et al*, 2005). White blood cell counts above 5/µl of CSF or protein levels above 25 mg percent are indicative of the CNS involvement. However, trypanosomes have been found in CSF even when cell counts were below the above levels (Molyneux and Ashford, 1983, Chappuis *et al*, 2005).

1.2.6 Chemotherapy of HAT

Chemotherapy is the main stay in the control of HAT due to antigenic variation of the blood stream trypanosomes which renders the parasites elusive to the immune response. Therefore, use of vaccines is still impossible and hence chemotherapy remains the only alternative.

Chemotherapy is the use of drugs to rid an infected host of parasites. Chemoprophylaxis is the use of the same or other drugs to prevent transmission and limit pathological effects of the disease. Chemotherapy is the most widely used approach and in many African countries, it is the only method used for control of HAT.

The search for trypanocidal compounds began early in the 20th century and the main impetus was provided by various developments in the organic chemistry of new synthetic dyes and other scientific disciplines and by the need to combat the serious epidemics of HAT raging in Africa at that time (Jordan, 1986). In 1902 the first *in vivo* model became available and experimental treatment of trypanosome infections was carried out. Out of a large number of preparations tested by Paul Ehrlich, only trypan red and arsenic compounds were found to be highly effective. Therefore chemotherapeutic research progressed mainly in two lines: the synthetic dyes and related compounds and the organic arsenicals (Keiser, 1999).

The prospect of achieving a cure by chemotherapy is close to 100% if the central nervous system is not involved. As the disease progresses the chances of success decrease (progressively). However, under good hospital conditions patients in the second stage of the disease stand a reasonable chance of being cured (Jordan, 1986).

Currently only four drugs are available for the treatment of HAT: Suramin (first used in 1922) and pentamidine (1940) are used for the treatment of the first stage, melarsoprol (Freidheim, 1949) and eflornithine (1990) for the second stage of disease (WHO, 1998).

1.2.6.1 Drugs for Treatment of HAT

1.2.6.1.1 Pentamidine (Pentamidine isethionate)

Pentamidine (Figure 5) is an aromatic diamidine which does not cross the blood-brain barrier and hence is used for treatment of the early phase of the disease. Pentamidine was introduced as a trypanocide in 1941 and is the drug of choice against *T. b. gambiense* infections. Though effective against *T. b. rhodesiense* infections, the efficacy is much more variable and primary resistance has been reported in some areas. The recommended dose for treatment is 2-4 mg of pentamidine isethionate per kg of body weight given intramuscularly in a total of 7 injections daily or on alternating days (Jordan, 1986).

Trypanosomes take up pentamidine via membrane transporters although other routes of entry may exist (de Koning and Jarvis, 2001). Although pentamidine has poor penetration through

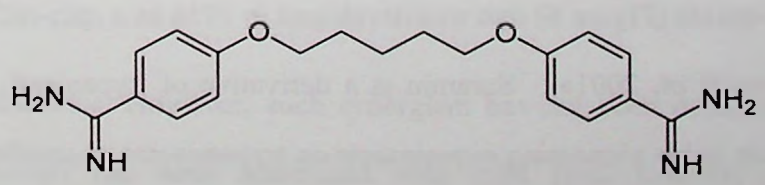

Figure 5: Pentamidine. Pentamidine is drug of choice against the first stage *T. b. gambiense* sleeping sickness.

the blood brain barrier, there are old observations that it can be successfully used to treat first stage II trypanosomiasis. The mode of action of diamidines remains uncertain (Sands *et al* 1985; Peregrine and Mamman, 1993) and therefore its trypanocidal effect may reflect the sum of many non-specific actions. Owing to their charge and hydrophobicity, diamidines have been likened to polyamines in their ability to interact with negatively charged intracellular components like nucleic acids and membrane phospholipids. Pentamidine forms tight complexes in the minor groove at AT-rich regions of double-stranded DNA. Ribosomal aggregation, inhibition of DNA and protein synthesis, and loss of the kinetoplast have been observed following drug exposure (Barrett and Fairlamb, 1999). In vitro inhibition has been reported for enzymes like mitochondrial topoisomerase II (Shapiro, 1993), plasma membrane Ca^{2+}-Mg^{2+} ATPase (Benaim *et al*, 1993) and S-adenosylmethionine decarboxylase (Bitonti *et al*, 1986). However, inhibition of S-adenosylmethionine decarboxylase by pentamidine does not appear to play a significant role in *vivo* (Berger *et al*, 1993) because earlier findings show that Pentamidine-sensitive trypanosomes exposed for 4 h *in vivo* to therapeutic doses of pentamidine (4 mg/kg) did not show any significant changes in either polyamine-, thiol- or S-adenosylmethionine metabolites, indicating that inhibition of S-adenosylmethionine decarboxylase is not involved in the trypanocidal action of the drug (Berger *et al*, 1993). Generally, it is believed that there is more than one target since diamidines accumulate to high concentrations in trypanosomes before death (Barrett and Fairlamb, 1999)

1.2.6.1.2 Suramin

Suramin is an aromatic oligo-amide (Figure 6) that was developed in 1916 as a spin-off of the German dye industry (Matovu *et al*, 2001a). Suramin is a derivative of trypan red, a dye originally used by Paul Ehrlich in his pioneering experiments on trypanosome chemotherapy. It was first tested against HAT in 1921 and is the drug of choice for use in *T. b. rhodesiense*

infection. The drug is administered intravenously at a dosage of 20 mg/kg and it has a long elimination half-life of 44-45 days. It is almost completely bound to plasma proteins (99%) and excreted predominantly via urine (Collins *et al*, 1986). It is used only against the first stage disease due to its inability to cross the blood-brain barrier (Wellde *et al*, 1989).

Like pentamidine, suramin's mode of action involves more than one target and hence its trypanocidal effect is a sum of many (non-specific) actions. It is reported to inhibit glycerol-3-phosphate oxidase and dehydrogenase. Suramin is also reported to inhibit the uptake of low-density lipoproteins with the consequent hampering of cell division (Matovu 200, Institute of Cell Biology, University of Bern; Barrett *et al*, 2007).

Although suramin poorly penetrates the CNS, it has been used as pre-treatment of the second stage disease, to reduce the toxicity of melarsoprol or to "sterilize" the patients until melarsoprol is administered. In animal studies, suramin has also been shown to have synergistic effects with other trypanocidal drugs like efflornithine, nifurtimox and 5-

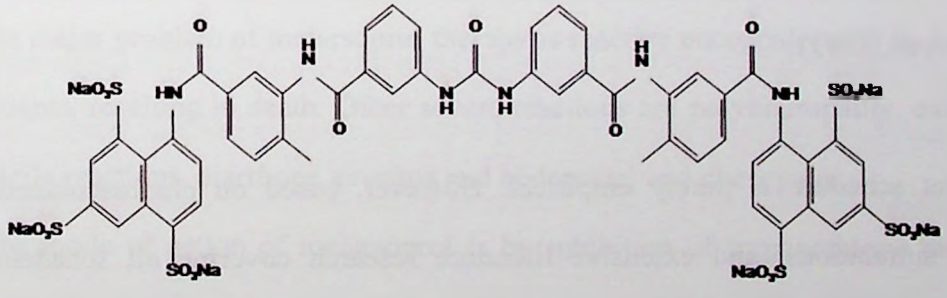

Figure 6: Suramin. Suramin is used against the first stage *T. b. rhodesiense* sleeping sickness

nitroimidazoles. However, such synergism has not been demonstrated in humans. Suramin monotherapy has been associated with mild renal toxicity, neuropathy and stomatitis. (Docampo and Moreno, 2002).

1.2.6.1.3 Melarsoprol

Melarsoprol (Figure 7) has been in use for treatment of HAT since 1949. It is very effective in the treatment of both the early and the second stage of HAT. However, due to the risks associated with its use, the drug is used only for the treatment of the second stage of the disease.

Melarsoprol is insoluble in water and is marketed as a 36 g/l solution in propylene glycol. Therefore, to prevent tissue damage by the highly toxic propylene glycol, the drug is administered intravenously (Matovu, 2001). The terminal half-life of the drug is 35 hours as determined by a biological assay (Burri *et al*, 1993) and the total protein binding is 79% as determined by ultrafiltration (Keiser and Burri, 2000). Studies performed in urine showed that melarsoprol was eliminated from the body only to a limited extent and hence to avoid accumulation of arsenic in the body, treatment interruptions after the 3rd or 4th injection were introduced (Apted, 1957). The maximum dose for each injection is 3.6 mg/kg. The treatment used to comprise several series of injections, each series separated by at least one week. Treatment may be associated with an acute reactive encephalopathy in 5 - 10 % of the cases (Ashford and Molyneux, 1983).

The above treatment schedule is purely empirical. However, based on pharmacokinetic analysis, computer simulations, and extensive literature research covering all schedules previously used and tested, a new treatment schedule consisting of 10 daily consecutive doses of 2.16 mg/kg was suggested (Burri *et al*, 1993). In a pilot clinical trial in Congo RDC using the new schedule, a non-significant increase of minor adverse reactions was observed, no case of reactive encephalopathy occurred and no relapse was reported in this group after 12 months (Burri *et al*, 1995). In another pilot study carried out in Kwanza Norte, Angola to assess the

safety and efficacy of the new schedule (10 daily injections of 2.2 mg/kg), it was found that the number of deaths and of patients with encephalopathic syndromes was the same as that observed with the standard schedule (three series of four daily injections at doses increasing from 1.2 to 3.6 mg/kg, with 7-days interval between series) but skin reactions were more common with the new schedule. However, considering the economic and practical advantages of the new 10-day schedule over the standard 26-day treatment schedule, and the similarity of the treatment outcome, the new schedule is a useful alternative especially in epidemic situations and in locations with limited resources (Burri *et al*, 2000).

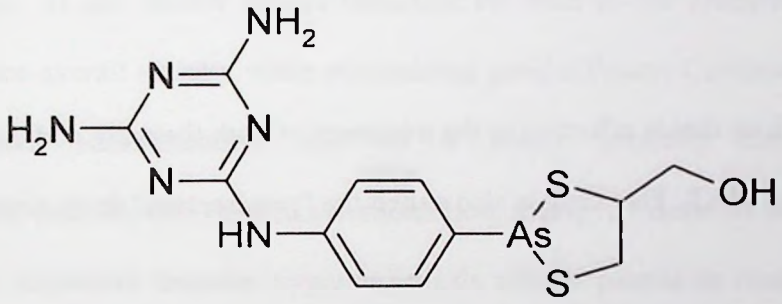

Figure 7: Melarsoprol. Melarsoprol is used against the second stage *T. b. gambiense* and *T. b. rhodesiense*, sleeping sickness.

The major problem of melarsoprol therapy is reactive encephalopathy in 5-10% of the treated patients, resulting in death. Other severe reactions are polyneuropathy, exfoliative dermatitis, febrile reactions, diarrhoea, pruritus and abdominal and chest pain.

The mode of action of melarsoprol is by inhibition of trypanothione reductase as primary target (Fairlamb *et al*, 1989). Trypanothione reductase is a key enzyme in the protection of trypanosomes against oxidative stress. Another mechanism has been suggested to be inhibition of phosphofructokinase, a key enzyme in the glycolytic pathway (Cunningham *et al*, 1994). The arsenical is thought to accomplish the inhibition by reacting avidly and reversibly with neighboring pairs of sulphhydryl groups in the enzymes (Barrett and Fairlamb, 1999).

1.2.6.1.4 Diflouromethylornithine

Diflouromethylornithine (DFMO) or Eflornithine is an ornithine analogue that irreversibly inhibits the enzyme ornithine decarboxylase (EC 4.1.1.17), the first enzyme in the synthesis of polyamines, essential in cell division and in the protection of the trypanosome against oxidant stress (Bellofatto *et al*, 1987; Hunter *et al,* 1991). DFMO was first synthesized in 1978 (Metcalf *et al*, 1978) and initially used for treatment of cancer (Bacchi *et al*, 1980). It is the first new trypanocidal drug since the introduction of melarsoprol and remains the only back-up drug for treatment of *T. b. gambiense* patients who relapse after melarsoprol treatment (Burri and Brun, 2003).

DFMO is a newly developed drug that is effective in the treatment of both the early and the second stage of *T. b. gambiense* HAT. This drug is also called the "resurrection" drug, since

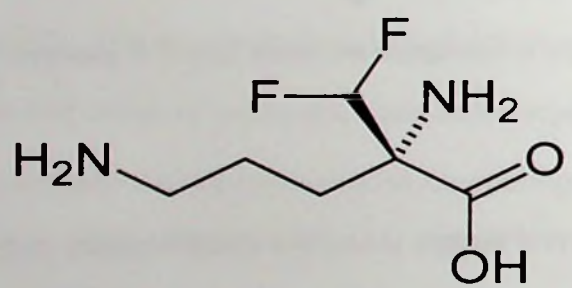

Figure 8: Difluoromethylornithine (DFMO). DFMO is used against seconda stage *T. b. gambiense* sleeping sickness.

comatous patients treated with Eflornithine may wake up rapidly and resume their activities. However, due to rapid excretion of the drug, the compound has to be given in very large quantities with a full treatment taking 400 grams of Eflornithine over a total period of two weeks, mainly as an infusion. Therefore, though relatively safe, it is less affordable than

melarsoprol, suramin and pentamidine (Matovu *et al*, 2001a) and poses immense logistic problems. In addition, DFMO is not effective in the treatment of *T. b. rhodesiense* infections.

1.2.6.2 Combination therapy for HAT

Combination therapy refers to the simultaneous administration of two or more medications to treat a single disease (http://en.wikipedia.org/wiki/Combination_therapy). Combination therapy has the potential to protect the drugs in the combination against selection of resistant strains (Barrett *et al*, 2007) and consequently less money needed for development of new drugs. It also allows dosage reduction for each of the drugs in the combination and hence reduce overall toxicity while maintaining good efficacy. Combination therapy may allow for a simpler administration and as a result improve the feasibility of treatment (http://en.wikipedia.org/wiki/Combination_therapy; Priotto *et al*, 2006, Pepin, 2007). This is very important because trypanosomiasis affects people in rural areas where health centres have limited logistics and staffing (Priotto *et al*, 2006).

A large number of animal studies have documented the potential synergism between various trypanocidal drugs. In HAT, several studies have been done both in laboratory animals and HAT patients. Combinations of Eflornithine and standard trypanocides like suramin and melarsoprol were found to be effective against acute *T. b. rhodesiense* infections in laboratory animals (Bacchi, *et al*, 1994).

Experiments have indicated synergism between eflornithine and melarsoprol even in melarsoprol-relapse patients. The aim of such a combination is to reduce on the amount of eflornithine needed and hence more drug available for melarsoprol-relapse patients. In

addition, this combination helps to reduce the toxicity due to melarsoprol monotherapy since less drug is used (Mpia and Pépin, 2002).

In Uganda, a randomized trial was done with three drug combinations; melarsoprol-nifurtimox, melarsoprol-eflornithine, and nifurtimox-eflornithine. The objective was to compare the efficacy and safety of the three drug combinations. Cure rates for the three drug combinations were 44.4%, 78.9% and 94.1%, respectively. Although results from this study could not permit conclusive interpretations, the nifurtimox-eflornithine combination appeared to be a promising first-line therapy for the second stage HAT (Priotto *et al*, 2006).

Another randomized clinical trial with nifurtimox-eflornithine combination was done in Congo with results showing that nifurtimox-eflornithine is a promising therapy for second stage HAT. However, studies from additional sites are needed before this combination is recommended (Priotto *et al*, 2007).

1.2.6.3 Treatment Relapse

A relapse occurs when a person is affected by a condition that affected them in the past (http://en.wikipedia.org/wiki/Relapse). In HAT management, relapses after treatment with first stage drugs (pentamidine and suramin) are rare except in cases where early second stage disease is misdiagnosed as first stage and the patient is given treatment for first stage (Brun *et al*, 2001). In treatment of second stage *T. b. gambiense* HAT, relapse rates after melarsoprol therapy of up to 18-20% (Ibbe, southern Sudan), 27% (northwestern Uganda) and 25% (northern Angola), have been reported (Pépin and Mpia, 2005).

In *T. b. gambiense* HAT management, it is however, not easy to say if the relapse is due to re-infection or treatment failure since during follow-up (18 months) it is possible that the patient

is bitten by an infected tsetse fly. This requires that microsatellite genotyping be done before treatment and when one relapses to differentiate between the trypanosomes that caused the previous infection and those causing relapse. However, this is not generally possible since HAT affects people in the rural areas where there may not be competent staff to do the genotyping unless when scientific research with such an aim is done.

In general terms, relapses are also referred to as treatment failures, refractoriness or unresponsiveness. Reasons for treatment failure could be host-related, parasite-related, drug – related, or related to disease management (Figure 9).

The immune status of an infected host plays a very important role in successful chemotherapy. For example (treatment of HAT with melarsoprol and more so for) DFMO is trypanostatic, and hence requires appropriate antibody-mediated immune responses (Berger and Fairlamb, 1992). Successful treatment with such drugs requires an intact immune system. Therefore, a competent immune system is necessary for successful chemotherapy. Presence of concurrent diseases is also an important factor. HIV, malaria, intestinal helminthes and protozoa, and filariasis are the most common concurrent infections that may be found in a HAT patient and therefore their management is very important. Another host-related factor is nutrition. It is shown that stress factors like malnutrition play an important role in the disease process (Atouguia and Costa, 1999; www.vet.uga.edu/VPP/gray_book02/fad/aat.php) and hence could be important in successful treatment. Host metabolism is another reason that plays an important role because it can result in altered pharmacokinetics (Brun *et al*, 2001). Metabolism is important where the active compound is not the native drug but its metabolite or where the drug is presented as a complex

Parasite-related factors include non-responsiveness of parasites to recommended doses. If parasites are resistant to the recommended dose, then the infection will survive the treatment. This may occur due to natural tolerance (innate lack of susceptibility to a drug) of the parasites to a given drug and does not require previous exposure to the drug. For example *T. b. rhodesiense* is innately refractory to DFMO (Brun *et al*, 2001). Drug resistance is another parasite-related factor. This occurs when parasites become resistant to a drug they were previously sensitive to. This can also be referred to as dosage failure where the parasites are resistant to the recommended dose but circum at higher doses (http://en.wikipedia.org/wiki/Drug_resistance).

Drug pharmacokinetics can also play a role in chemotherapeutic failure. Drug pharmacokinetics refers to a number of features that influence the fate of the drug over a period of time post-administration. These include absorption, distribution, metabolism, and excretion of the drug. While some drugs are rapidly absorbed from the site of application and attain peak plasma levels within a short time other drugs are slowly absorbed. For example in dogs, diminazene aceturate was reported to reach peak levels within 10-15 minutes and to be excreted to below detection levels within 48 hours following intramuscular administration of 3.5 mg/kg (Alui *et al,* 1993). On the other hand, isometamidium was reported to be slowly absorbed from tissues at the site of injection and was detected from the same tissues, liver and kidney 6 weeks after treatment (Kinabo and Bogan, 1988). Some drugs partly or do not completely access the CNS or some compartments of the host, hence treatment failure occurs if such drugs are used in infections that involve the CNS or such compartments (Jennings *et al,* 1979). Use of expired drugs can also cause treatment failure because an expired drug is most likely less potent and not safe.

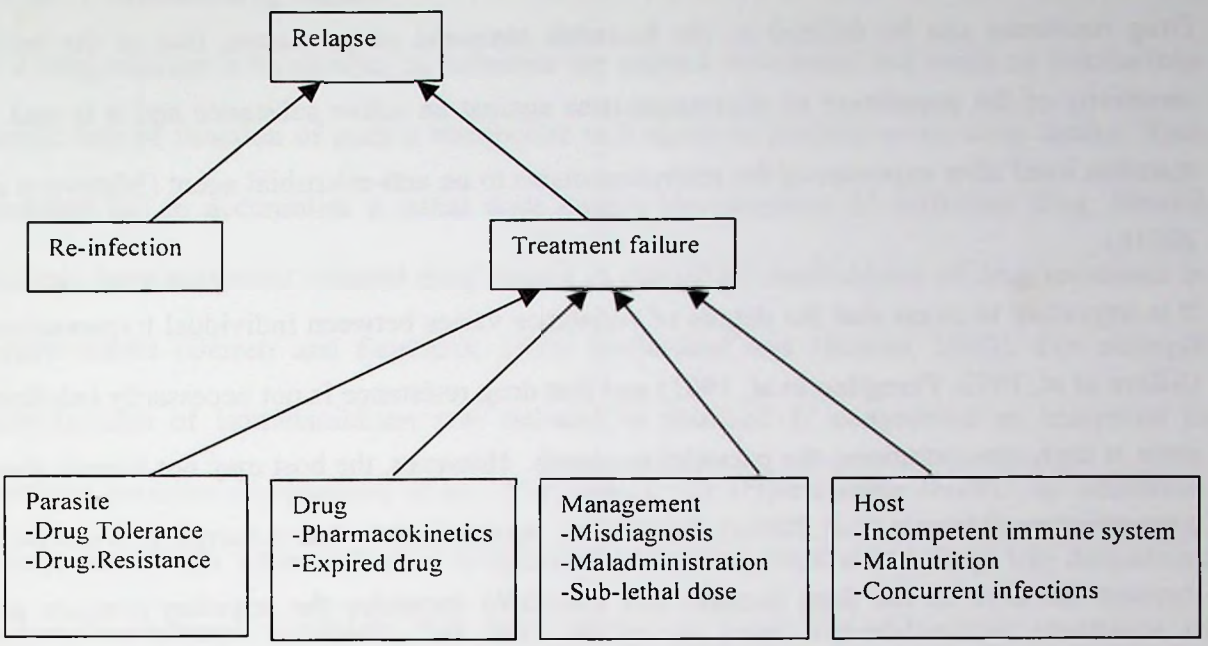

Figure 9: Reasons for treatment failure or relapses. Solid arrows indicate causal relationship.

Factors related to disease management include misdiagnosis of disease, application of sub-lethal dose and maladminstration of the drug. Misdiagnosis can occur for example in early second stage HAT which could be diagnosed as first stage. Most health centres do not have the diagnostic tools or competent staff to make correct diagnosis and staging of the disease. As a result, an early second stage patient is treated as a first stage and yet drugs for the latter do not cross the blood brain barrier. (Atouguia and Costa, 1999; Kaminsky *et al*, 1990). Sub-therapeutic drug concentrations provide an ideal environment for the selection of resistant parasites that pre-exist in the trypanosome population (Matovu *et al*, 2001a; Besier and Hopkins, 1988). Maladministration of the drug which refers to the use of a wrong route since different routes of administration are recommended for different drugs. Therefore, selecting a wrong route can cause failure.

1.2.6.4 Drug Resistance in Trypanosomes

Drug resistance can be defined as the heritable temporal or permanent loss of the initial sensitivity of the population of microorganisms against an active substance and it is said to manifest itself after exposure of the microorganisms to an anti-microbial agent (Matovu *et al*, 2001b).

It is important to stress that the degree of resistance varies between individual trypanosomes (Silayo *et al*, 1992; Peregrine *et al*, 1991) and that drug resistance is not necessarily indefinite since at high concentrations, the parasites succumb. However, the host may not tolerate these concentrations (Matovu *et al*, 2001a). In addition, once resistance has occurred, it is unwise to increase the dose of the drug because this inevitably increases the selection pressure and hence the level of resistance (Silayo *et al*, 1992).

1.2.6.5 Mechanisms of drug resistance in *Trypanosoma brucei*

There are various means parasites use to elude toxic effects of the drug despite presence of required drug levels (Matovu *et al*, 2001a). Understanding the mechanisms parasites use to elude the effect of drugs is important because it can lead to the identification of potential and novel drug targets and provide direction to how new chemotherapeutic strategies can be used to reduce the development of resistance.

Experiments done by different scientists have shown, reduced net drug uptake (reduced drug accumulation) to be a frequent mechanism of trypanocidal drug resistance. Reduced net drug uptake can be caused by either reduced drug import or increased drug export and both mechanisms imply mutations in the transporter since most trypanocides can not freely diffuse through the plasma membrane (Mäser *et al*, 2003).

1.2.6.5.1 Reduced drug import

If a drug requires a transporter to permeate the plasma membrane and reach an intracellular target, loss of function of such a transporter will result in reduced or no drug uptake. Such parasites fail to accumulate a lethal dose despite the presence of sufficient drug. Several findings have suggested reduced drug import as one of the mechanisms of drug resistance in trypanosomes (Barrett and Fairlamb, 1999; Sutherland and Holmes, 1993). For example accumulation of isometamidium was reduced in resistant *T. congolense* as compared to sensitive parasites (Sutherland, *et al*, 1991; 1992). In *Trypanosoma brucei*, an adenosine transporter TbAT1 which is known to transport adenosine, adenine and drugs like diamidines and melarnophenyl arsenicals, has been shown to have loss-of-function mutations in laboratory selected drug resistant trypanosomes (Mäser *et al*, 1999). In the field, TbAT1 alleles with loss-of-function mutations were shown to be significantly more prevalent in trypanosomes from melarsoprol-relapse *T. b. gambiense* patients than in newly-infected ones. (Matovu *et al*, 2001b). However, the observations that, the *tbat1* null mutant was 15-fold resistant to berenil, only two to three-fold more resistant to melarnophenyl arsenicals and pentamidine (Matovu *et al*, 2003) and that adenosine did not inhbit the uptake of pentamidine and melarsen in the null mutant, confirmed the existence of alternative adenosine-insensitive import pathways for the two drugs (Carter *et al*, 1995; Scott *et al*, 1997; de Koning 2001). This was further strengthened by findings that pentamidine inhibited melarsen-induced lysis of the *tbat1* null mutant; an indication that a second transporter exists for both drug (Matovu *et al*, 2003). More about the role of TbAT1 in drug resistance in *Trypanosoma brucei* is discussed in chapters 3A, 3B and 3C.

1.2.6.5.2 Increased drug export

Drug export is effected by ATP-binding cassette (ABC) transporters. ABC transporters couple ATP hydrolysis to substrate translocation and hence are able to pump their substrate against a concentration gradient. Active drug export by P-glycoproteins (subset of ABC transporters) has been reported in some kinetoplastids and other parasites (Wilson *et al,* 1993; Quellette and Papadopoulou, 1993; Orozco *et al*, 1995). ABC transporters have been identified in *T. brucei* and although their involvement in drug resistance in the field has not been found (Matovu *et al*, 2001b; Barrett and Fairlamb, 1999), laboratory experiments have shown that overexpression of TbMRPA, one of the ABC transporters in *T. brucei,* in the laboratory causes melarsoprol resistance (Shahi *et al*, 2002; Chapter 3A).

1.2.7 New Drug Targets in *Trypanosoma brucei*

One way to manage drug resistance is to identify new targets (Mäser, December, 2008). Current drugs used for treatment of HAT have been available for more than a century, have significant side effects and the failure rate for melarsoprol is high (Naula *et al*, 2005). Therefore there is urgent need for new drugs against *Trypanosoma brucei subsp.* A new drug will need to meet a number of criteria. For example it should be able to cross the blood brain barrier so as to be effective against the second stage disease when the parasites are found in the CNS, they should also be effective against both *T. b. rhodesiense* and *T. b. gambiense*, be effective against melarsoprol-resistant trypanosomes and there should be minimal propensity for the parasites to become resistant. Finally, it should be cheap since the disease affects the poorest people in the world (Lüscher *et al*, 2007).

Research aimed at identifying and validating novel drug targets is a major focus of the kinetoplastid community (Naula *et al*, 2005). Approaches like mRNA differential display,

microarray analysis and two dimensional gel analysis (2DGE) have been used in identifying proteins that are essential and specific to parasites (Hua and Wang, 1994; Ehigie, 2006)

However, with the availability of fully sequenced genomes, novel antiparasitic drug targets may be identified *in silico*. Differential genomics approaches can be used to compare genomes of parasites and their host. Genes found to be present in the parasites but absent in their hosts can be taken as potential drug targets which can be validated through experimental procedures like gene knockout and RNAi (Naga and Ravichandran, Naula *et al*, 2005; Lüscher *et al*, 2007). One of such postgenomic approaches is by analyzing metabolic networks (Chapter 4). This approach is a step towards facilitating the search for new drugs targets.

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Chapter 2.0

Comparative genomics and Serodiagnostic evaluation of Tandem Repeat Proteins in *Trypanosoma brucei.*

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Abstract

Human African Trypanosomiasis (HAT) is a neglected disease with respect to treatment as well as diagnostics. In the absence of laboratory equipement under field conditions, serology is still the mainstay for HAT diagnosis. However, the current tests are either not sensitive enough or not specific enough and hence a need for more antigens to be evaluated. Here we implement an *in silico* pipeline for the identification of *Trypanosoma. brucei*-specific Tandem Repeat (TR) proteins. Repeat-containing proteins were retrieved from the Dora database (http:/genomics.unibe.ch/dora) and sequentially blasted against proteomes of *Homo sapiens* and pathogens common in areas where HAT is endemic. The antigenicity of the 19 candidate proteins were predicted using different computational methods which showed that 17 proteins were highly antigenic while two were not. Three candidate genes were PCR amplified, cloned and expressed in *E. coli*. The recombinant proteins were then purified and evaluated using ELISA using serum from HAT patients. Preliminary results show that the three selected recombinant proteins are not good diagnostic antigens since they do not discriminate between sleeping sickness patient sera and sera of healthy individuals. Since the number of sera used for ELISA is few, these proteins will be further evaluated by FIND Diagnostics (Geneva) before conclusive remarks can be made about them.

Introduction

Trypanosoma brucei is the causative agent of African trypanosomiasis. *Trypanosoma brucei* constitutes three subspecies; *Trypanosoma brucei rhodesiense*, *Trypanosoma brucei gambiense*, and *Trypanosoma brucei brucei*, *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* are the causative agents of the acute and chronic, forms of Human African Trypanosomiasis (HAT) or sleeping sickness, respectively. On the other hand, *Trypanosoma brucei brucei* is the causative agent of nagana in cattle (Stich *et al*, 2002, Matovu *et al*, 2003; Chappuis *et al*, 2005).

Chemotherapy is the principle control method for trypanosomiasis since antigenic variation of the parasites rules out the possibility of vaccination (Matovu *et al*, 2003; Alibu *et al*, 2006). Treatment of cases depends on accurate diagnosis of the disease. Field diagnosis of trypanosomiasis involves both direct and indirect methods (Connor R. J. FAO, 1992). Direct methods are based on parasite detection and they include microscopic examination of lymph node aspirates, blood and celebrospinal fluid (CSF) (Stich *et al*, 2002; Connor R J, FAO, 1992; Chappuis *et al* 2005). However, this requires a high degree of training and expertise. In addition, as the concentration of the trypanosomes in the blood undulates due to antigenic variation, parasite detection may be difficult especially in *T. b. gambiense* infections where concentrations often decrease below detection levels (Stich *et al*, 2002; Coronor R J, FAO). On the other hand, indirect methods are based on demonstrated presence of specific antibodies in the blood, plasma or serum of infected hosts. These include tests like the Card Agglutination Test for Trypanosomiasis (CATT) and the LATEX/*T. b. gambiense*, used in diagnosis of the *T. b. gambiense* infections (Chappuis *et al*, 2005). Although CATT is a sensitive assay, specificity remains a problem because of cross-reactivity with antibodies to closely related non-pathogenic trypanosomes and antibody titres do not distinguish between past and current infections (Agranoff *et al*, 2005, Chappuis *et al*, 2005). The LATEX/ *T. b. gambiense* was developed as a field alternative to CATT. The LATEX/ *T. b. gambiense* test is based on the combination of three purified surface antigens; Litat 1.3, 1.5, and 1.6. It showed a higher specificity but lower or similar sensitivity in recent field studies. Further evaluations are needed before it is recommended for routine field use. As mentioned earlier, both CATT and LATEX/*T. b. gambiense*, are restricted to *T. b. gambiense* infections (Chappuis *et al*, 2005). There is therefore need for tests that are sensitive, specific and can detect infections due to all the three *T. brucei subsp.*

Tandem repeat (TR) proteins containing two or more copies of an amino acid pattern have been found in many organisms and are known targets of B-cell epitopes. TR proteins have been found to have immunological significance during bacterial infections and in cancer. Many virulence factors from parasites contain tandem repeats e.g the circumsporozoite protein of *P. falciparum*. Furthermore, in protozoan parasites, TR proteins often serve as targets of B-cell responses. For example, in Chagas disease, Leishmaniasis and Malaria, antibody responses to TR proteins, have been found (Dame *et al*, 1984; Koenen *et al*, 1984, Coppel *et al*, 1984; Allred *et al*, 1990; Burns *et al*, 1992; Burns *et al*, 1993; Gruber and Zingales, 1993; Bhatia *et al*, 1999). Although *Trypanosoma brucei* has many TR genes (Goto *et al*, 2007, Fankhauser *et al*, 2007), very little has been done to evaluate the serodiagnostic potential of TR proteins. In a study done by Müller and colleagues, two TR proteins; GM6 and MARP1 were found to have immunologic sensitivity of up to 90% and hence interesting candidates for immunodiagnosis of trypanosomiasis in cattle (Müller *et al*, 1991). However, the function and immunological role of these proteins is still not clear. The aim of this work was to identify and to evaluate the serodiagnostic potential of TR proteins in the diagnosis of sleeping sickness.

We have previously developed a tool for genome-wide surveys of TR proteins called Dora (database of repetitive antigens) (http:/genomics.unibe.ch/dora/) in our group (Fankhauser *et al*, 2007). We performed a computational search for TR genes using the Dora and the proteins obtained were then sequentially blasted against the proteomes of *L. major, H. sapiens, P. falciparum* and *E. coli,* to eliminate proteins that are of higher sequence identities, which may cross react. The candidate genes were PCR amplified, cloned and expressed in *E. coli.* The expressed proteins were then purified and used for ELISA using serum from sleeping sickness patients.

Materials and Methods

1. Repetitive protein gene identification

We used the Dora database (http:/genomics.unibe.ch/dora/) (Fankhauser *et al*, 2007) to identify repetitive proteins of *Trypanosoma brucei* irrespective of whether they carried GPI anchors, Transmembrane domains, N-terminal export signal or N-glycosylation with an e-value smaller than 10e-10. For the subsequent filtering processes, perl scripts were written. First and foremost, proteins with less than nine (9) amino acid residues in a repeat were removed. The remaining proteins were subjected to a sequential-blasting procedure to get rid

of proteins that have higher sequence identities with proteins in *H. sapiens* and in common organisms causing diseases in the same regions where the trypanosomiasis is prevalent. A flow diagram of the sequential blasting procedure is shown in Figure 1. The resulting proteins with low or no identities were blasted against EST databases or GeneDB, for information concerning their expression in bloodstream form parasites. Hydrophilicity (Kyte/Doolittle and Hopp/Woods, methods) and Antigenicity (Parker antigenicity, Protrusion index antigenicity, Welling antigenicity and antigenic index, methods) predictions of the candidate proteins were performed using the MacVector 10.6.0 version. In general, the antigenicity prediction methods are based on the assumption that antigenic determinants would be exposed on the surface of the proteins and hence should be hydrophilic (Hopp and Woods, 1981; Kyte and Doolittle, 1983; Welling *et al*, 1985; Hopp, 1986; Thornton *et al*, 1986; Parker *et al*, 1986; Jameson and Wolf, 1988). Three of those proteins found to be to be very hydrophilic, highly antigenic by atleast three of the methods used and expressed in bloodstream forms (using available data in at www. genedb.org at the time of experiments), were taken as candidates for experimentation.

2. PCR and cloning of repetitive protein genes

Partial or complete TR domains containing multiple repeat units were amplified by PCR from genomic DNA from either only *T. brucei brucei* or both *T. brucei brucei* and *T. brucei rhodesiense*. TR domains of Tb09.211.1800, Tb10.406.0650 (MARP1) and Tb10.70.6570, were PCR amplified using primer sets from *T. b. rhodesiense* gDNA or plasmid DNA. Tb09.211.1800 primers (TB091800F and TB091800R) anneal outside the repeat region and gDNA from both *T. b. rhodesiense* laboratory strain and *T. b. rhodesiense* field strain, were used. Two primer sets were used for Tb10.70.6570; Tb706570forward1 anneals to the non-repetitive region at the 5`end while the corresponding reverse primer Tb706570reverse1 anneals within the repeat region. On the other hand Tb706570forward2 anneals within the repeat region while the corresponding reverse primer anneals outside the repetitive region at the 3` end. Tb10.70.6570 was amplified from the *T. b. brucei* laboratory strain. Only a very small fragment of MARP1 equivalent to one repeat unit (about 100bp) could be amplified from gDNA using gene-specific primers. Therefore the MARP1 fragment used for expression was amplified from an existing pBSMARP1 plasmid. The amplified MARP1 fragment is much bigger than the insert. With the help of the flanking restriction sites, the insert was restricted and recloned in pTOPO PCR2.1 vector (Invitrogen). Primers used for amplification of all the genes are shown in Table 2. Note that all primer sets have BamHI restriction site in

forward primer and XhoI restriction site in the reverse primer, as indicated in bold. The resulting PCR fragments were cloned in pTOPO PCR2.1 vector (Invitrogen) and sequenced using the BigDye mix terminator v3.1 cycle sequencing kit. Midi preparations were made from cultures of colonies with the correct inserts.

3. Expression and purification of recombinant proteins

Plasmid DNA preparations with Tb09.211.1800 and Tb10.70.6570, inserts were digested using BamHI and XhoI, while plasmid DNA with MARP1 insert was digested using EcoRI, for 3 hours at 37^0C. The digested products were run on agarose gels and bands of correct size were cut out and gel-purified. The purified fragments were then inserted in-frame with the six His tag of the Parallel expression vectors (Sheffield, 1999) which were then transformed into the *Escherichia coli* BL21 (DE3) strain. The transformed cells were grown in yeast extract-tryptone medium and when OD_{600} had reached 0.6, expression of the proteins was induced with 1mM Isopropyl-β-D-thiogalactopyranoside for 3 hrs. The cells were harvested and lysed by sonication 6x 15sec at 120mA. After centrifugation, the pellet was used to purify inclusion bodies from Tb09.211.1800 and Tb10.406.0650 (MARP1), proteins following the procedure of Uli Baumann (personal communication). The purity of the inclusion bodies was assessed by Coomassie blue staining following SDS-PAGE. Inclusion bodies were not prepared from Tb927.70.6570 protein because it was soluble in the LEW buffer (50 mM Sodium phosphate and 300 mM Sodium chloride) used for sonication.

Protein purification was done using the Talon metal affinity resin (Ref 635502, BD Biosciences Clontech, 1020 E. Meadow Circle Palo Alto, Ca 94303). Tb10.70.6570 protein was purified under native conditions while Tb09.211.1800 and Tb10.406.0650, proteins were purified under denaturing conditions. Proteins were eluted using 250 mM imidazole Elution buffer. A sample of each of the purified proteins was run on a 12% SDS-PAGE gel to assess their purity. Protein concentrations were determined using the Commassie Plus™ Protein Assay (The Better Bradford Assay).

4. Patient sera.

Human African Trypanosomiasis patient sera were collected from patients with active disease (Provided by Dr Enock Matovu, Makerere University, Kampala, Uganda). Sera of healthy individuals from, sleeping sickness endemic areas (provided by Johannes Blum, STI

Basel) and Switzerland (provided by Barbara Seebeck, Bern, Inselspital), were used as negative controls.

5. Enzyme-Linked Immunosorbent Assay (ELISA)

Optimal conditions were determined empirically, resulting in the following procedure.

Proteins were diluted in 1x PBS to a concentration of 5 µg/ml and 50 µl were dispensed into each well in an ELISA plate (Coster 3690, High Binding). After coating overnight at 4^0C, the coating solution was discarded and the plate was washed twice with 1x PBS. From this point to the end of the experiment all steps were done at room temperature and all incubations with shaking. 180µl of blocking buffer (PBS/0.5% BSA) were added to each well and incubation was done for 2hrs. After blocking, blocking buffer was aspirated, 50 µl of patient or control sera, diluted 1:100 in blocking buffer, were added to their respective wells while blank wells received 50 µl of blocking buffer instead of 50 µl of sera, and incubation was done for 2 hrs. The plates were washed three times with PBS/0.1% tween20 and three times with PBS. 50 µl of Horseradish peroxidase (HRP)-conjugated anti-human IgM, diluted 1: 1000 in blocking buffer were then added to each well and again incubation was done for 2hrs. The plates were again washed three times with PBS/0.1% tween20 and three times with PBS before adding 50 µl of developing solution (containing 3 3`5 5`Tetramethylbenzidine and Hydrogen peroxide, in citrate buffer). After incubating for 15 min with developing solution, the reaction was stopped by adding 50 µl 0.5 M H_2SO4 and OD was read at 450nm using an ELISA reader (SPECTRAMAX 340PC, Bucher Biotec AG. 4051 Basel). Results were analysed using Prism Graph Pad Software version 4.0a.

Results

Tandem Repeat Protein Identification

Of the predicted *T. brucei* proteome (approx. 9100 proteins), 121 met the criteria of carrying a repeat length greater than 9 and p-value below 10^{-10}. These hits were sequentially blasted against proteomes of *L. major, H. sapiens, P. falciparum*, and *E. coli*, as shown in Figure 1. Nineteen (19) proteins were obtained and information regarding their expression profiles and repeat analysis is shown in Table 1. Note that the order of blast searches does not influence which protein remains in the end. Hydrophilicity (Kyte/Doolittle and Hopp/Woods) and Antigenicity (Parker antigenicity, Protrusion index antigenicity, Welling antigenicity and antigenic index, methods) predictions of the candidate proteins were performed using the MacVector 10.6.0 version. Of the 19 candidate proteins, 17 were predicted to be highly

hydrophilic by both methods and contain antigenic domains, while two (Tb11.0080 and Tb11.1320) were predicted to be hydrophobic and do not contain antigenic domains. Overall, the Protrusion index antigenicity and the Parker antigenicity, methods predicted the entire sequence of the repetitive proteins to be antigenic while Welling antigenicity and Antigenicity Index, methods show antigenicity to be predominant at specific sites rather the entire repetitive sequence.

PCR and Cloning

PCR amplification was done from *T. b. rhodesiense* gDNA in the case of Tb09.211.1800 (from both field and laboratory strains) and Tb10.70.6570, and pBSMARP1 plasmid in the case of Tb10.406.0650. Primers used are shown in Table 2. Tb09.211.1800 gene from *T. b. rhodesiense* laboratory strain is larger than *T. b. rhodesiense* field strain (Figure 2) with a difference of about 300bp. The MARP1 fragment amplified from pBSMARP1 is much bigger than the insert because it contains the flanking restriction sites. The amplified MARP1 fragment was therefore cloned in pTOPO PCR 2.1 from which the insert was restricted and recloned in pTOPO PCR2.1. In the case of Tb10.70.6570, several fragments were amplified since one primer of each primer set could anneal at many regions due to the repetitiveness of the gene (Figure 3). However, only a fragment of about 400bp was used. All inserts were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit to ensure they were the right ones.

Expression and purification

The selected proteins were expressed as recombinant His-tagged proteins in *E. coli* BL21 (BD3) and purified using the Talon metal affinity resin. Tb09.211.1800 and Tb01.406.0650, recombinant proteins were purified under denaturing conditions while Tb10.70.6570 recombinant protein was purified under native conditions. Expression and purification results are shown in figure 4.

Enzyme-Linked Immunosorbent Assay (ELISA)

Twelve (12) sleeping sickness patient sera, ten (9) sera from healthy individuals from sleeping sickness endemic areas and two (16) sera from healthy individuals from Switzerland, were used to examine the prevalence of antibodies against the four recombinant proteins. ELISA was done in the following order. First it was done with 12 sleeping sickness sera and one negative control from Switzerland. All the proteins showed significantly stronger

reactivities to the patient sera as compared to the negative control. However, when the nine (9) negative controls from Sleeping sickness endemic area were used, there was no significant difference between OD readings of the patient sera and those of the negative controls from sleeping sickness endemic areas. Results are summarized in Figure 5. We furthered evaluated the proteins with 16 sera of individuals from Switzerland to find out if there is a difference in reactivities between sera of healthy individuals in sleeping sickness endemic areas and that of individuals in Switzerland. Results obtained show that there is no significant difference between the two groups.

Discussion and Conclusions

Serological tests are important for diagnosis and mass screening of *T. b. gambiense* sleeping sickness. The aim of this work was therefore to identify *T. brucei*-specific proteins that can be used as antigens to develop a serological test. Tandem repeat (TR) proteins are known B-cell epitopes and several studies has shown that TR proteins are highly antigenic (Müller *et al*, 1991; Imboden *et al*, 1995; Goto *et al*, 2006, 2007, 2008). In this study we retrieved extremely repetitive proteins from *T. brucei* genome v4 using the Dora database (Fankhauser *et al*, 2007) using parameters described in methods above. The 509 proteins which were retrieved were subjected to subsequent filtering as indicated in Figure 1 so as to obtain proteins that are *T. brucei*-specific. 19 candidate proteins were obtained with the above criteria. All the candidate proteins except Tb11.0080 and Tb11.1320, were predicted to be highly hydrophilic and contain antigenic domains. Tb11.0080 and Tb11.1320, were predicted to hydrophobic in agreement with the fact that these proteins are mainly composed of non-polar amino acids (Leucine and Isoleucine). The difference in antigenicity predicted by Parker antigenicity and Protrusion index antigenicity, and Welling antigenicity and Antigenicity Index, methods, illustrates the need to use different methods since use of only one method would not give adequate information. Three of those found to be highly antigenic and expressed in blood stream parasites (hydrophilicity and self-alignment plots are shown in Figure 2), were selected for expression in *Ecoli* BL21 (DE3) as his-tagged recombinant proteins. The purified proteins were evaluated for serodiagnostic potential using ELISA. Results obtained show that although the antigens are well recognized by sleeping sickness patient sera, they also cross-react with sera of both healthy individuals from sleeping sickness endemic areas (Figure 4) and negative control sera from Switzerland (results not shown). This is not a completely new phenomenon since earlier studies showed that TR proteins I/6 and MARP1 were recognized by self-reactive antibodies from uninfected mice (Müller *et al*,

1992; Detmer *et al*, 1996). However, in our study it is not known if the cross-reactivity is due to self-reactive antibodies or antibodies against related epitopes of other pathogens. It is also possible that some of the individuals from sleeping sickness endemic areas assumed to be healthy were in their very early stages of the disease and the antibody levels were too low to be detected with the available test. In conclusion, although TR proteins are highly antigenic, they are not always specific and hence the need for more TR proteins to be evaluated so as to get those that do not cross-react. In addition, *in silico* methods need to be supplemented with laboratory experimentation so as to obtain parasite-specific proteins that can be used as antigens to develop a diagnostic test. Therefore *in silico* predictions serve as initial filters that help to identify potential antigens which are then validated using laboratory experimentation. Finally, in our study, the number of sera used was too small to make conclusive remarks about above TR proteins. We have therefore submitted these proteins to FIND-DIAGNOSTICS (Geneva, Switzerland) for further evaluation with a bigger number of sera and in parallel with other known antigens.

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Figures and Tables

Figure 1

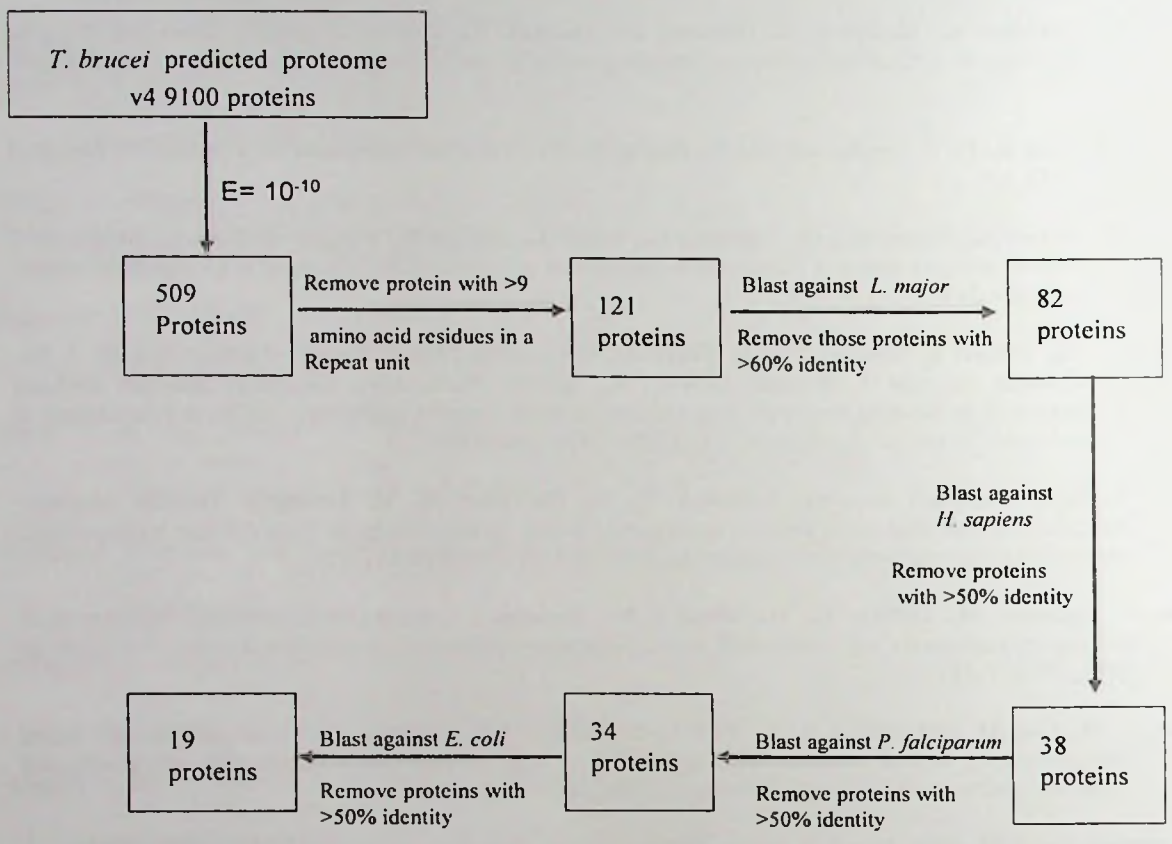

Figure 1: Repeat protein retrieval and sequential blasting procedure for identifying *Trypanosoma brucei*-specific repetitive proteins.

Table 1

Trypanosoma brucei-specific proteins obtained by the sequential blasting procedure.

Protein ID	Description	Protein size	*Repeat size	Repeat number	Expression data
Tb927.1.1740	hypothetical	7154	40	132	BSF[1]
Tb11.0080	hypothetical	210	35	2	No data
Tb927.8.7060	hypothetical	1578	40	17	BSF[2]
Tb927.2.5860	hypothetical	1242	29	18	No data
Tb09.211.1800	Membrane-associated, hypothetical	495	24, 8	8, 12	BSF[2, 3], PCF[3]
Tb10.406.0650	Microtubule associated protein, putative	2257	37	47	BSF[2, 3, 4], PCF[3, 5]
Tb10.61.0560	hypothetical protein, conserved	1748	175	3	BSF[2]
Tb927.7.3830	kinesin K39, putative	1803	39	25	BSF[3], PCF[3]
Tb927.7.4130	hypothetical protein, conserved	2408	61	7	No data
Tb10.70.6570	Membrane-associated, hypothetical	2872	78	14	BSF[2]
Tb11.1320	hypothetical	658	40	3	No data
Tb10.70.7300	hypothetical	1502	146	2	PCF[5]
Tb11.01.8770	hypothetical	1004	11	5	BSF[2, 3], PCF3[3,5]
Tb11.02.0210	hypothetical	461	10	5	BSF[2, 3, 4], PCF[3]
Tb927.2.5870	hypothetical	1992	32	10	BSF[2]
Tb927.4.1160	hypothetical	908	9	32	No data
Tb927.4.2070	hypothetical	4455	15, 7	66, 102	BSF[2, 3, 6], PCF[3, 6]
Tb927.7.2650	hypothetical protein, conserved	537	43	2	BSF[2, 3], PCF[3]
Tb927.8.3870	hypothetical protein	1205	28	23	BSF[2, 3], PCF[3]

1. Subramaniam *et al*, 2006, 2. Bridges *et al*, 2008, 3 Vertommen *et al*, 2008, 4. Broadhead *et al*, 2006, 5. Jones *et al*, 2006, 6. Koumandou *et al*, 2008; *This column indicates only perfect repeats which gave lowest p-value, the protein may contain more repeats than those indicated. Repeat analysis: http://reptile.unibe.ch, Fankhauser *et al*, 2007

Figure 2

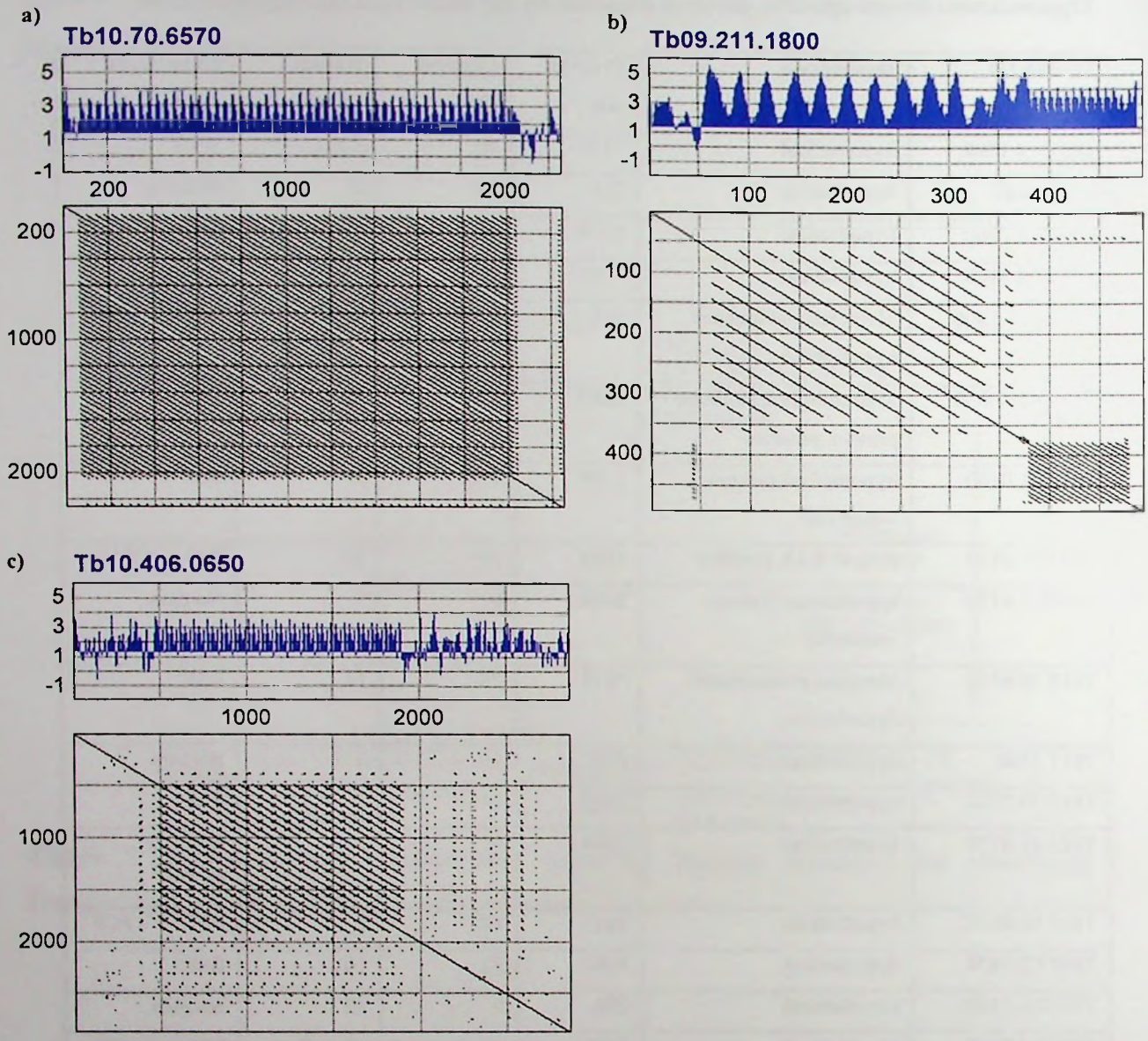

Figure 2: Hydrophilicity plots (in blue above) and self-alignment plots (below), of the three selected proteins. a) Tb10.70.6570, b) Tb09.211.1800 and c) Tb10.406.0650. They were plotted using MacVector version 10.6.0. Hydrophilicity is used as a measure of protein antigenicity.

Table 2:

Table showing DNA source and primers used for amplification of the selected *T. brucei* TR proteins

Protein	DNA used	Primer annealing region
Tb09.211.1800	*T. b. rhodesiense* lab strain	TB091800F 5`GCCC**GGATCC**CTGAGAGAGTCGAACTACATC`3` TB091800R2 5`GCC**CTCGAG**TTATCGCTGTCATCGAACCTTG3`
Tb09.211.1800	*T. b. rhodesiense* field strain	TB091800F 5`GCCC**GGATCC**CTGAGAGAGTCGAACTACATC3` TB091800R2 5`GCC**CTCGAG**TTATCGCTGTCATCGAACCTTG3`
Tb10.406.0650	pBSMARP1 Plasmid (Schneider *et al*, 1992)	pBSMARP1-forward 5`GCCCGCTGGAATTCGCCCTTGGATAC3`, pBSMARP1-reverse 5`GCCCAGAATTCGGCCCTTAGCTTGATCTGG3`
Tb10.70.6570		Tb706570forward1 5`GCCC**GGATCC**GAGGTAGACTCGTTGCGTC3`, Tb706570reverse1 5`GCC**CTCGAG**TTACTGTCTGAGAAGTTCATCG3` Tb706570forward2 5`GCCC**GGATCC**ATTCTTGAACGCCTTAATG3` Tb706570reverse2 5`GCC**CTCGAG**TTAAACACCACAATATCCACG3`

Figure 3

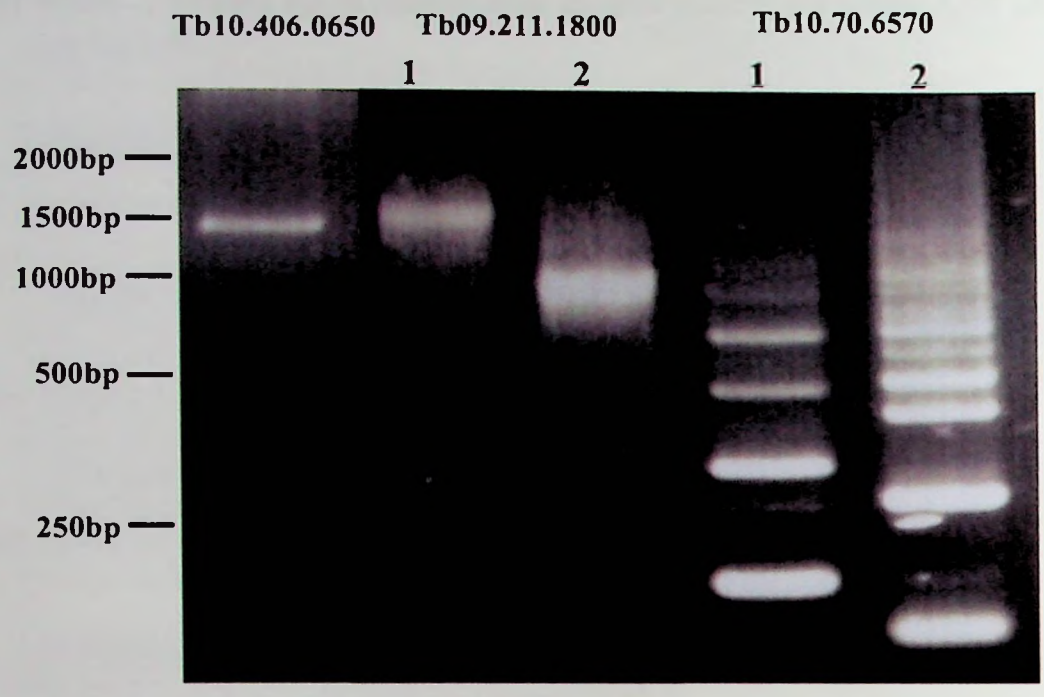

Figure 3: PCR analysis of *Trypanosoma brucei* TR genes. Tb10.70.6570 and Tb09.211.1800 were amplified from *T. b. rhodesiense* genomic DNA while Tb10.406.0650 was amplified from pBSMARP1 plasmid. Details of the source of DNA and primers, are shown in Table 2.

Figure 4

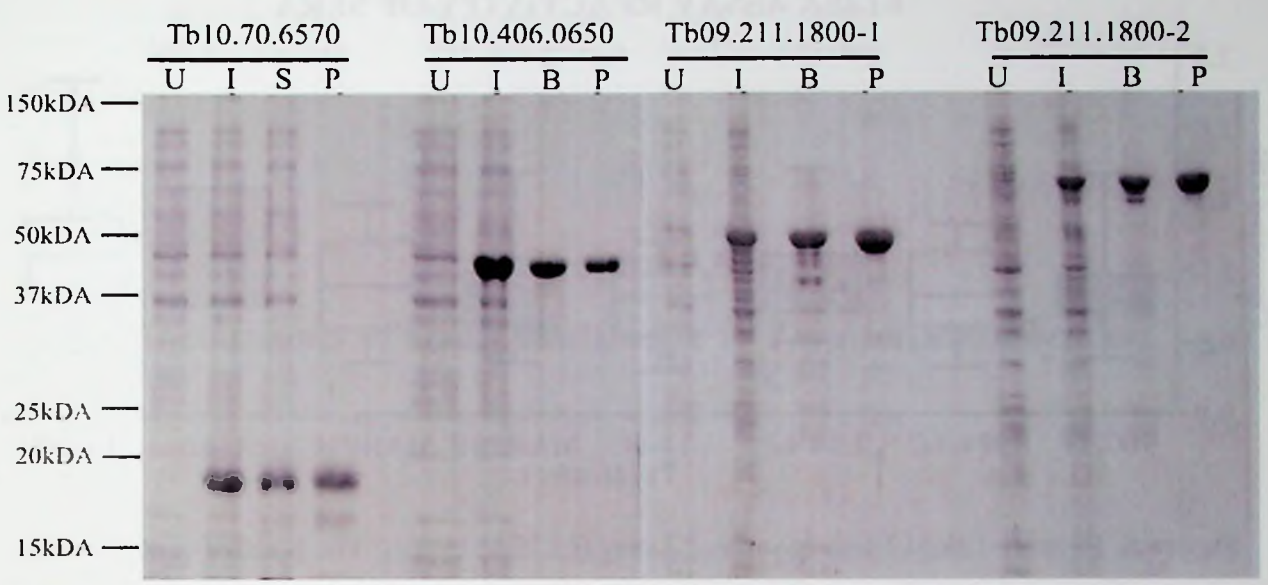

Figure 4: Protein purification was done using the Talon metal affinity resin. Tb10.70.6570 (16-6) was purified under native conditions while the rest were purified under denaturing conditions. Tb09.211.1800-1 was expressed from *T. b rhodesiense* field strain DNA (L14-6) while Tb09.21.1800-2 was expressed from *T. b. rhodesiense* laboratory stain (900-1). U= Uninduced, I= Induced, S=Supernatant, B= Inclusion bodies, P= Purified proteins.

Figure 5

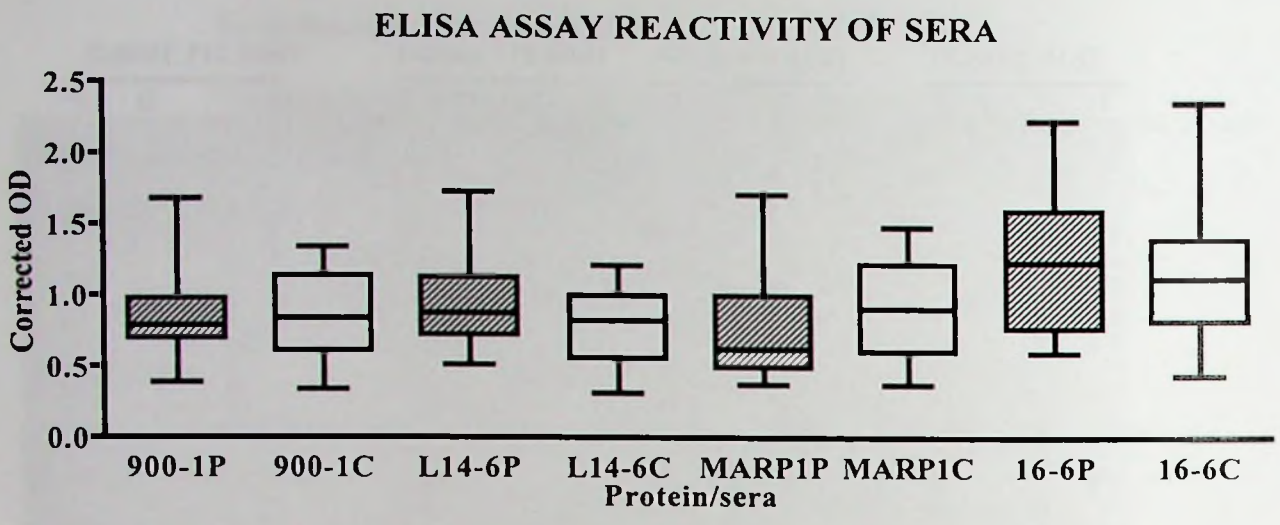

Figure 5: Enzyme-linked Immunosorbent Assay (ELISA) results. The purified proteins were used as antigens for ELISA using Sleeping sickness patient sera. Sera from nine (9) healthy individuals from Sleeping sickness endemic areas were used as negative controls. 900-1P, L14-6P, MARP1P and 16-6P represent reactivities of the respective proteins with patient sera (shaded boxes) while 900-1C, L14-6C, MARP1C and 16-6C represent reactivities of the respective proteins with negative controls from sleeping sickness endemic areas (open boxes). There is no significant difference between reactivities of the different proteins with patient sera and those with negative control sera.

Chapter 3.0

Mechanisms of Drug Resistance in *Trypanosoma brucei*

Chapter 3.0a

Combined contribution of TbAT1 and TbMRPA to drug resistance in *Trypanosoma brucei.*

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I generated the NY-mrpa line and performed the western blotting and all the long incubation tests (Alamar Blue Assays).

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Short communication

Combined contribution of TbAT1 and TbMRPA to drug resistance in *Trypanosoma brucei*

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Melarsoprol is a melamine-based arsenical that was developed against sleeping sickness by the Swiss chemist Ernst Friedheim in 1949. Melarsoprol would never pass today's safety trials, causing encephalopathy in 5–10% of treated patients [1]. However, it is still the only drug approved for treatment of late-stage sleeping sickness that is active against both *Trypanosoma brucei rhodesiense* and *T. b. gambiense*. While melarsoprol relapse rates of around 5% are considered normal, alarmingly high incidences of 20–30% treatment failures were reported from sleeping sickness foci in Uganda, the Democratic Republic of Congo, and the Sudan [2]. Two possible mechanisms for resistance against melaminyl arsenicals are known at the molecular level: decreased drug influx by loss of the adenosine transporter TbAT1, and increased drug efflux by overexpression of the ABC transporter TbMRPA. Both mechanisms reduce net cellular drug uptake, a phenomenon already described for arsenical resistant trypanosomes by Hawking 70 years ago [3].

TbAT1 encodes the P2 aminopurine transporter, responsible for cellular uptake of a remarkable collection of trypanocides including melaminyl arsenicals, pentamidine, diminazene aceturate, isometamidium, cordycepin and tubercidin [4–9]. Homozygously disrupted *tbat1* knock-out trypanosomes exhibited different degrees of resistance towards these agents; only between two- and three-fold to melarsoprol and melarsen, indicative of additional uptake pathways for melaminyl arsenicals [10]. Nevertheless, loss-of-function point mutations in *TbAT1* were described from melarsen-resistant *T. b. brucei* lab mutants as well as from *T. b. gambiense* field isolates, and the occurrence of such mutations correlated to a certain degree with melarsoprol treatment failure [5,11].

TbMRPA was initially called TbABC1 [12] and renamed because it belongs to the *m*ultidrug-*r*esistance-associated-*p*roteins [13], subfamily C of the *A*TP-*b*inding *c*assette (ABC) transporters. MRP proteins are able to actively extrude drug–glutathione complexes from the cytosol. TbMRPA is thought to function as an efflux pump of trypanothione conjugates [13], as was demonstrated for the closely related LtMRPA from *Leishmania tarentolae* (formerly known as LtPgpA [14,15]). Trypanothione is made of two glutathione moieties linked by spermidine [16]. Simultaneous overproduction of LtMRPA and γ-GCS (γ-glutamylcysteine synthetase, the rate-limiting enzyme of glutathione synthesis), caused 16-fold arsenical resistance in a particular *L. tarentolae* strain while either gene overexpressed alone only reduced the sensitivity by a factor of 2 [17]. In *T. b. brucei*, overexpression of TbMRPA by itself decreased melarsoprol susceptibility by factors from 5 to 10 [13,18]. However, concomitant overproduction of γGCS and ODC (ornithine decarboxylase, the dedicative enzyme in spermidine synthesis) had a negligible effect, suggesting that in contrast to *Leishmania*, trypanothione supply is not limiting for arsenical detoxification in trypanosomes [13]. At least not under standard cell culture conditions, in vivo the situation may be different [18].

Here we test what happens if both known mechanisms for melarsoprol resistance, loss of TbAT1 and gain in TbMRPA, coincide in one cell. Can we obtain high-level resistance to melaminyl arsenicals in *T. brucei* by simultaneously decreasing drug influx and increasing drug efflux? To answer this question we made three isogenic, transporter-manipulated lines of the New York single-marker *T. b. brucei* bloodstream forms [19]: line NY-at1^{-} was homozygously disrupted in *TbAT1* by consecutive replacement of either allele with puromycin and phleomycin resistance genes; line NY-mrpa^{+} was stably transformed with linearized plasmid pHD1057 [13], where *TbMRPA*

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is under control of the Tet promoter; and the double-mutant line NY-at1^{-}mrpa^{+} lacked *TbAT1* and overexpressed *TbMRPA* at the same time. All NY lines constitutively express T7 RNA polymerase plus the Tet repressor [19]. NY-mrpa^{+} and NY-at1^{-}mrpa^{+} transformants of comparable TbMRPA expression levels under induction with tetracycline were selected based on Western blots (not shown). All further experiments with these lines were performed in the presence of 1 μg/ml tetracycline.

Melarsen susceptibility of the four NY lines was first measured with a simple lysis assay [20], where trypanosomes are incubated at micromolar drug concentrations and cell lysis is monitored by the decrease in optical density; a typical experiment is shown in Fig. 1A. The transporter-mutated lines all showed a delay in melarsen-induced lysis compared to parental cells. This effect was most pronounced for the double mutant NY-at1^{-}mrpa^{+}. The single mutant lines had intermediate phenotypes, with NY-at1^{-} consistently being more resilient to lysis than NY-mrpa^{+} (Fig. 1A). The situation was somewhat reversed at more physiological, nanomolar drug concentrations: in low inoculation, long incubation tests ("Alamar blue assays" [21], example shown in Fig. 1B), line NY-mrpa^{+} was significantly more resistant to melarsen than NY-at1^{-} (Fig. 1C). The finding that overexpression of *TbMRPA* gave the stronger resistance phenotype in long-term assays than deletion of *TbAT1* is in agreement with previous reports [10,13]. The highest degree of melarsen resistance was again exhibited by NY-at1^{-}mrpa^{+} double mutant trypanosomes (Fig. 1B and C). With an IC_{50} of 66 nM melarsen oxide, they can hardly be called high-level resistant though. As summarized in Table 1, the contributions from loss of TbAT1 and gain in TbMRPA were strictly independent.

For further phenotyping of the three transporter-mutated *T. b. brucei* lines, they were subjected to trypanocides of different permeation pathways. The experimental compound phenylarsine oxide is an aromatic arsenical that, due to its hydrophobicity, freely diffuses across membranes [22]. Once in the cytosol, it is likely to be complexed to trypanothione and expelled via TbMRPA as well. Phenylarsine oxide was even more trypanotoxic than melarsen oxide, with an IC_{50} of 3 nM against the parental *T. b. brucei* NY line. Overexpression of *TbMRPA* reduced phenylarsine sensitivity by about two-fold. This was observed for both NY-mrpa^{+} and NY-at1^{-}mrpa^{+} when compared to parental NY and NY-at1^{-} trypanosomes, respectively (Fig. 1C, Table 1), and in both cases the difference was highly significant ($p < 0.001$, Newman–Keuls multiple comparison test). On the other hand, the presence or absence of *TbAT1* had no influence on phenylarsine sensitivity (Fig. 1C), which was not surprising since phenylarsine lacks amidine groups and is not a P2/TbAT1 substrate.

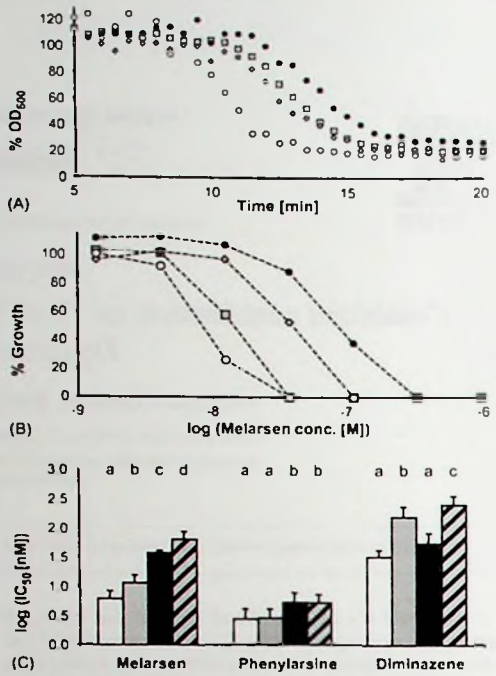

Fig. 1. Drug sensitivities of transporter-mutated *Trypanosoma brucei brucei* bloodstream forms. (A) For lysis assays, 10^6 cells were incubated at 37 °C in 200 μl PBS, 1 mM glucose, 15 μM melarsen oxide. (B) For Alamar blue assays [21], 1000 cells in 200 μl HMI9 medium were incubated in serial dilutions of melarsen oxide for 72 h, cell growth was measured with the redox-activated dye Alamar blue (white, NY; grey squares, NY-tbat1^{-}; grey diamonds, NY-mrpa^{+}; black, NY-tbat1^{-}mrpa^{+}). (C) Selected drugs were tested at least seven times, each in duplicate, and IC_{50} values were determined by non-linear regression. Error bars indicate standard deviation, small letters represent significance groups for each drug as determined by one-way ANOVA plus Newman–Keuls post-test (white, NY; grey, NY-tbat1^{-}; black, NY-mrpa^{+}; striped, NY-tbat1^{-}mrpa^{+}).

Diminazene, in contrast, is a trypanocidal diamidine that enters bloodstream-form *T. brucei* predominantly via P2/TbAT1 [9]. Diminazene aceturate (berenil) is widely used for the treatment of Nagana. RNAi-mediated gene silencing of *TbAT1*

Table 1
IC_{50} in nM and resistance factors (R) of parental (NY) and transporter-mutated *Trypanosoma brucei brucei* bloodstream forms as determined by Alamar blue assays [21]

	NY-at1^{-}		NY-mrpa^{+}		NY-at1^{-}mrpa^{+}		$(R_{at1^-} \times R_{mrpa^+})/R_{at1^-mrpa^+}$
	IC_{50}	R	IC_{50}	R	IC_{50}	R	
Melarsen	11.9	1.9a	37.2	5.8a	66.1	10.3a	1.04
Phenylarsine	3.0	1.0b	5.6	1.9b	5.5	1.8b	1.03
Diminazene	168	5.0c	58.1	1.7b	264	7.9c	1.11

Letters indicate significance groups within each mutant (one-way ANOVA on each column, Newman–Keuls post-test; for comparison between the mutants see Fig. 1). Last column: the product of the single mutants' resistance factors equaled the double mutant's resistance factor for all drugs tested.

caused berenil resistance in *T. evansi* [23]. The IC_{50} of diminazene aceturate against the parental NY line was 33 nM. Diminazene resistance from loss of *TbAT1* was clearly apparent in line NY-at1$^-$ (Fig. 1C), albeit to a smaller extent than described previously in another *T. b. brucei* background [10]. To our surprise, also overproduction of TbMRPA lowered the susceptibility to diminazene (Fig. 1C, Table 1). The effect was small, about 1.7-fold when comparing NY-mrpa$^+$ to NY, or NY-at$^-$mrpa$^+$ to NY-at1$^-$ (Fig. 1C), but in the latter case highly significant ($p < 0.001$, Newman–Keuls multiple comparison test). Apparently the phenotype from overexpression of *TbMRPA* is more readily scored in a *tbat1* null background. Cross-resistance between melaminyl arsenicals and diamidines in *T. brucei* spp. is a well-known phenomenon [24], for which the unusual substrate specificity of P2/TbAT1 provided a possible explanation [4]. Our results indicate that TbMRPA may be involved as well. The ABC transporter PRP1 from *L. major*, 32% identical to TbMRPA, caused resistance to pentamidine and antimony(III) chloride when overexpressed in promastigote leishmania [25].

The question what happens when different resistance mechanisms coincide is important since in pathogenic bacteria or tumor cells, drug resistance is often caused by interplay of several mutations. Here we show that there is no risk for high-level resistance to arsenicals by concomitant overexpression of *TbMRPA* and loss of *TbAT1* in bloodstream-form *T. b. brucei*. The two transporters contribute independently to drug resistance, and the effects from combination of the two mutations were strictly additive for all drugs tested (respectively multiplicative when considering resistance factors, Table 1). Whether high-level arsenical resistance can occur after additional, synergistic mutations remains to be investigated. Candidate mechanisms include loss of additional drug uptake transporters such as HAPT1 [10], or post-translational modifications as described recently by Foucher et al. [26].

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Chapter 3.0b

Melarsoprol- and pentamidine-resistant *Trypanosoma brucei rhodesiense* populations and their cross-resistance.

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I performed DNA extraction and molecular characterization of the *TbAT1* gene.

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Melarsoprol- and pentamidine-resistant *Trypanosoma brucei rhodesiense* populations and their cross-resistance

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Abstract

Resistance to melarsoprol and pentamidine was induced in bloodstream-form *Trypanosoma brucei rhodesiense* STIB 900 in vitro, and drug sensitivity was determined for melarsoprol, pentamidine and furamidine. The resistant populations were also inoculated into immunosuppressed mice to verify infectivity and to monitor whether rodent passage selects for clones with altered drug sensitivity. After proliferation in the mouse, trypanosomes were isolated and their IC_{50} values to the three drugs were determined. To assess the stability of drug-induced resistance, drug pressure was ceased for 2 months and the drug sensitivity was determined again. Resistance was stable, with a few exceptions that are discussed. Drug IC_{50}s indicated cross-resistance among all drugs, but to varying extents: resistance of the melarsoprol-selected and pentamidine-selected trypanosomes to pentamidine was the same, but the pentamidine-selected trypanosome population showed lower resistance to melarsoprol than the melarsoprol-selected trypanosomes. Interestingly, both resistant populations revealed the same intermediate cross-resistance to furamidine. Resistant trypanosome populations were characterised by molecular means, referring to the status of the *TbAT1* gene. The melarsoprol-selected population apparently had lost *TbAT1*, whereas in the pentamidine-selected trypanosome population it was still present.

Keywords: Resistance; Cross-resistance; Trypanosome; Furamidine; Melarsoprol; Pentamidine; *Trypanosoma brucei rhodesiense*; *TbAT1* gene

1. Introduction

Resistance to drugs is a major problem in the treatment of infectious diseases. A distinction can be made between innate and acquired resistance. Innate resistance (i.e. tolerance) is found when organisms of a given species are insensitive to treatment without ever having been in contact with the drug, whereas acquired resistance is the result of a mutation, conferring a selective advantage to the resistant organism over its sensitive siblings, depending on drug pressure. Ehrlich reported resistance of trypanosomes to fuchsin in 1907 (Ehrlich, 1907). However, little is known about the mechanism(s) of resistance. Identification of the P2 transporter and its recognition motif was a major breakthrough in the investigation of increasing resistance of trypanosomes to melarsoprol (Carter and Fairlamb, 1993; Mäser and Kaminsky, 1998; de Koning and Jarvis, 1999). When antiparasitic drugs act specifically against a target within the parasite, resistance may easily develop as a consequence of minor changes in this target. A target may be located inside the cell or on its surface. However, melarsoprol and pentamidine are likely to have several targets inside the cell (Barrett and Fairlamb, 1999). To date, resistance in trypanosomes to melarsoprol and pentamidine has been shown to originate from reduced drug uptake. Studies performed by de Koning with strain 247 trypanosomes (*Trypanosoma brucei brucei*) showed that pentamidine transport is mediated by three distinct transporters: the aminopurine transporter P2, the high affinity pentamidine transporter HAPT and the low affinity

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pentamidine transporter LAPT (de Koning, 2001b). The P2 transporter, encoded by the *TbAT1* gene (Mäser et al., 1999 and Matovu et al., 2003), plays a significant role in pentamidine uptake into trypanosome bloodstream forms, and is responsible for 50–70% of the total uptake (Carter et al., 1995; de Koning, 2001b). This transporter is also of major importance in melarsoprol uptake (Carter and Fairlamb, 1993). The uptake of melarsoprol is not only attributed to the P2 transporter but also to a yet unknown transporter, possibly HAPT1 (Matovu et al., 2003). Therefore, cross-resistance between diamidines and melaminophenyl arsenicals is to be expected, but as Bray et al. (2003) stated, not predictable. Since both drugs, pentamidine and melarsoprol, rely on the P2 transporter, mutations in *TbAT1* may lead to cross-resistance. Recent studies showed the P2 transporter to be the principal, but not exclusive, route for uptake of furamidine (Lanteri et al., 2006). The diamidine furamidine is the active metabolite of the prodrug pafuramidine, a newly developed orally available trypanocide which is presently undergoing clinical phase III trials. The involvement of the P2 transporter in furamidine uptake is not surprising, since the recognition motif can be found not only within the molecular structures of pentamidine and melarsoprol, but also in that of furamidine (Barrett and Fairlamb, 1999; de Koning and Jarvis, 1999).

Since genetic disruption of *TbAT1* only lead to a resistance factor of 2–3 to pentamidine and melarsoprol (Matovu et al., 2003), it can be assumed that in trypanosome populations selected for high-level resistance over a longer period of time, the loss of more than one transporter is involved in resistance. Drug resistance in pathogens in the field is mostly pleiotropic, i.e. acquired by accumulation of minor changes. Such changes are usually based on spontaneous mutations and emerge particularly in organisms with a short generation time and a high frequency of combinatorial events (Rang et al., 2003). High genetic exchange occurs mainly in bacteria. In trypanosomes, sexual recombination is known to occur only in the procyclic forms in the tsetse fly and not in bloodstream forms (Jenni et al., 1986). Generally, mutations leading to drug resistance deliver a selective advantage to the mutant over the wild type under drug pressure, but impose a certain cost on the parasites in the absence of drugs (Fohl and Roos, 2003). A recent study, however, showed that, in vitro, *TbAT1* knockout trypanosomes had a growth advantage over the wild type, possibly from overexpression of other, P1-type purine permeases (Geiser et al., 2005). The cost for melarsoprol resistance is not known, but mutations in the P2 transporter could lead to reduced uptake of adenine and adenosine.

One strategy to delay or prevent the development of resistance is to combine drugs that act on different targets and therefore exhibit different modes of action. Additionally, synergistic effects may arise from a combination of two drugs that act differently. In such a situation, the concentrations of the combination partners can be reduced compared with those used in a monotherapy, which leads to better safety. Whiteside (1958) suggested the use of so-called "sanative" pairs of drugs which preclude cross-resistance, e.g. isometamidium (Samorin) and diminazene aceturate (Berenil) against livestock-pathogenic trypanosomes. Melaminophenyl arsenicals and diamidines are what might be called "insanative" pairs of drugs, because cross-resistance between the two chemical classes has been observed repeatedly in African trypanosomes (Rollo and Williamson, 1951; Fulton and Grant, 1955; Frommel and Balber, 1987; Osman et al., 1992; Zhang et al., 1993; Pospichal et al., 1994; Barrett et al., 1995; Scott et al., 1997). This raised the question whether melarsoprol resistance in the field may be selected for by treatment with diamidines (Barrett, 2001). In order to further investigate the phenomenon of cross-resistance between melaminophenyl arsenicals and diamidines, we independently induced resistance to melarsoprol and pentamidine in *Trypanosoma brucei rhodesiense*. The sensitivity of the resistant populations to either drug is determined along with their cross-resistance to furamidine. The stability of the resistance (i) after in vitro cultivation of the trypanosomes in the absence of the drug and (ii) after propagation of the resistant trypanosome populations in the mouse, is also studied. The status of the P2 transporter in the resistant trypanosome populations is investigated by diagnostic PCR on *TbAT1*.

2. Materials and methods

2.1. Trypanosomes

Trypanosoma brucei rhodesiense STIB 900 is a derivative of STIB 704 which was isolated from a male patient at St. Francis Hospital in Ifakara, Tanzania, in 1982. After several passages in rodents and a cyclic passage in *Glossina morsitans morsitans*, a cloned population was adapted to axenic growth in vitro. This clonal strain serves as a reference strain in the drug screening and lead finding process.

2.2. Culture medium

Trypanosomes were cultivated as bloodstream forms in Minimum Essential Medium (MEM) with Earle's salts (powder; GIBCO, Invitrogen, Basel, Switzerland), supplemented with 25 mM *N*-2-Hydroxyehtylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (GIBCO), 1 g/L additional glucose, 2.2 g/L $NaHCO_3$ and 10 ml/L MEM non-essential amino acids (×100; GIBCO). The medium was further supplemented with 0.2 mM 2-mercaptoethanol, 2 mM sodium pyruvate, 0.1 mM hypoxanthine, 0.016 mM thymidine and 15% heat-inactivated horse serum (Baltz et al., 1985).

2.3. Drugs

Furamidine was synthesised by Dr. D.W. Boykin, Georgia State University, Atlanta, GA, USA (Das and Boykin, 1977). Melarsoprol was obtained from WHO, Geneva, Switzerland, donated by Sanofi-Aventis, and pentamidine

as Pentacarinat® from Rhone-Poulenc, Thalwil, Switzerland. Endoxan® (cyclophosphamide) was purchased from Baxter, Volketswil, Switzerland, and resazurin from Fluka, Buchs, Switzerland.

2.4. Drug sensitivity assay

Trypanosomes were tested in vitro for their sensitivity to melarsoprol, pentamidine and furamidine. They were incubated for 72 h in serial drug dilutions in a 96-well plate (Räz et al., 1997). After addition of the viability indicator resazurin (12.5 mg in 100 ml PBS) and a further incubation for 2–4 h, plates were read at excitation wavelength 536 nm and emission wavelength 588 nm using the fluorescence reader SpectraMAX Gemini XS (Bucher Biotech AG, Basel, Switzerland). To determine the IC_{50}, data analysis was performed with SoftmaxPRO 3.1.2.

The resistance factor was calculated by dividing the IC_{50} obtained for the resistant population by the IC_{50} of each compound obtained for the wild type. IC_{50} values represented in the tables are average values of three independently performed drug sensitivity assays, in which the determination of the IC_{50} was performed in duplicate.

A one way analysis of variance (ANOVA) and Tukey's post hoc test were performed to statistically assess the stability of resistance (significance threshold $P = 0.05$).

2.5. Propagation of resistant trypanosomes in immunosuppressed mice

Female Naval Medical Research Institute (NMRI) mice with a body weight of 18–22 g were purchased from RCC, Ittingen, Switzerland. Mice were immunosuppressed by applying 200 mg/kg Endoxan® i.p. Resistant trypanosomes were concentrated to 5×10^6 trypanosomes in 0.25 ml and were inoculated i.p. into mice. Tail blood was checked every second day for trypanosomes. When parasitaemia was high, stabilates were made and stored in liquid nitrogen. Trypanosomes which had been propagated in mice were tested for their drug sensitivity.

Animal use adhered to the guidelines issued by the Swiss Federal Veterinary Department (BVET) for laboratory animals. Mice were housed in individual cages in a temperature-controlled environment (20–22 °C) under a 12-h light-dark cycle. Standardised pellet food and tap water were available ad libitum. Experimental protocols were approved by the Cantonal Veterinary Department of the Canton Basel-Stadt.

2.6. Molecular characterisation of the TbAT1 gene

Genomic DNA was isolated from *T.b. rhodesiense* bloodstream forms grown in mice by phenol/chloroform extraction. *TbAT1* was amplified by PCR with Taq polymerase (Sigma–Aldrich, Basel, Switzerland) using primers ATF-1 (gaaagcttaatcagaaggatgctcgggtttgactca) and ATR-1 (gaggatcctgaacagtattcgtatgacgattagtgctac; annealing temperature 55 °C) which anneal to the 5′ and 3′ untranslated regions of *TbAT1*, or primers Sfa-s (cgccgcaacteatcgcccccgttt) and Sfa-as (ccaccgcggtgagacgtgta; 62 °C) which anneal in the coding sequence of *TbAT1* (annealing temperature 55 °C). Control primers specific for *T. brucei* spp. actin were act-s (ccgagtcacacaaacgt) and act-as (ccacctgcataaacattg; 55 °C). The Sfa-s/Sfa-as PCR product (677 bp) was digested with SfaNI (New England Biolabs, Bioconcept, Allschwil, Switzerland) and run on a 2.5% agarose gel. PCR products were cloned into pCR2.1 using the Topo-TA cloning kit from Invitrogen (Carlsbad CA, USA). Cycle sequencing was performed with BigDye Terminator v3.0 (Applied Biosystems, Foster City CA, USA).

3. Results

3.1. Melarsoprol- and pentamidine-resistant STIB 900 populations

Bloodstream-form *T.b. rhodesiense* STIB 900 were cultured with a subcurative dose of either melarsoprol or pentamidine, beginning with 7.5 nM melarsoprol and 1.7 nM pentamidine. After several subpassages, when proliferating trypanosomes no longer showed a difference in size, morphology or growth rate compared with trypanosomes cultured without drug, the drug concentration was increased stepwise by a factor of 2. Resistant populations were obtained after 18–24 months.

Trypanosomes grown in melarsoprol showed an IC_{50} of 187 nM to melarsoprol compared with the wild type STIB 900 population with an IC_{50} of 7.5 nM (Table 1; resistance factor: 24.9, Table 2). Pentamidine-selected trypanosomes showed an IC_{50} of 280 nM for pentamidine compared with an IC_{50} of 1.7 nM for the wild type (Table 1; resistance factor: 164.7, Table 2).

3.2. Cross-resistance

The melarsoprol-selected population showed a high cross-resistance to pentamidine (Table 2), whereas only an intermediate cross-resistance to melarsoprol was found

Table 1
Cross-resistance profiles of the drug-resistant *Trypanosoma brucei rhodesiense* populations

Population	Melarsoprol	Pentamidine	Furamidine
Wild type STIB 900	7.5 ± 1.7	1.7 ± 0.2	4.0 ± 1.7
Melarsoprol-selected	187 ± 42[a]	296 ± 12[a]	45 ± 15[a]
- after 60 days in culture	196 ± 7.5[a]	272 ± 48[a]	51 ± 20[a]
- after mouse passage	162 ± 28[a]	200 ± 57[a]	63 ± 26[a]
Pentamidine-selected	95 ± 33[a]	280 ± 27[a]	54 ± 26[a]
- after 60 days in culture	45 ± 12[b]	204 ± 36[b]	49 ± 21[a]
- after mouse passage	76 ± 22[a]	237 ± 25[a,b]	51 ± 19[a]

Values are IC_{50} ± S.D. in nM.
[a,b]Significant groups as determined by Tukey's post test from analysis of variance on each group of three values.

Table 2
Overall resistance factors (RF) of the drug-resistant *Trypanosoma brucei rhodesiense* populations

Drug	RF melarsoprol-selected population	RF pentamidine-selected population	*P* (*t*-test)
Melarsoprol	24.2	9.6	<0.0001
Pentamidine	151	141	0.50
Furamidine	13.2	12.8	0.85

in the pentamidine-selected population (Table 2). A *t*-test for independent samples showed a statistically significant difference between the IC_{50}s to melarsoprol of the melarsoprol and pentamidine-selected populations ($P < 0.0001$, Table 2). Cross-resistance to furamidine was intermediate in both the melarsoprol- and pentamidine-selected populations (Table 2).

3.3. Stability of resistance

Trypanosomes showing resistance were cultured without drug pressure. After 2 months (120 generation times), the sensitivity to melarsoprol, pentamidine and furamidine was determined. Melarsoprol-selected trypanosomes showed no significant change in sensitivity to any of the drugs (Table 1). Cultivation of the pentamidine-selected population in the absence of drug pressure resulted in an insignificant decrease of resistance for melarsoprol and a significant reduction in the resistance to pentamidine. However, no change in drug resistance was observed for furamidine (Table 1).

3.4. Selection in mice

Resistant trypanosomes inoculated into mice produced a moderate to high parasitaemia within a few days. Trypanosomes isolated from those mice underwent several passages in culture before they were tested for drug sensitivity. Resistance of the melarsoprol-selected trypanosome population to melarsoprol and furamidine was shown to be stable, whereas their resistance to pentamidine decreased slightly (Table 1). There was no loss of resistance to melarsoprol, pentamidine or furamidine when the pentamidine-selected trypanosome population was propagated in the mouse (Table 1).

3.5. Status of the TbAT1 gene

Genotyping of *TbAT1* was performed by the diagnostic PCR-restriction fragment length polymorphism (RFLP) method known to distinguish between resistant- and sensitive-type *TbAT1* alleles (Mäser et al., 1999; Matovu et al., 2001). Parental *T.b. rhodesiense* STIB 900 and their pentamidine-selected descendants both exhibited the *TbAT1* sensitive-type digestion pattern (Fig. 1). The PCR products (677 bp) were cloned, sequenced and found to be identical in parental STIB 900 and pentamidine-selected progeny (not shown). The melarsoprol-selected population, however, appeared to have lost the *TbAT1* gene. Neither primers in the coding region of *TbAT1* (Fig. 1) nor primers in the 5′ and 3′ untranslated regions of the gene (not shown) produced a PCR product. PCR amplification of *T. brucei* spp. actin served as a positive control.

4. Discussion

Cross-resistance between melaminophenyl arsenicals (such as melarsoprol) and diamidines (such as pentamidine) in laboratory strains has been reported earlier (Rollo and Williamson, 1951; Barrett et al., 2003). The mode of action of the two classes of drugs is assumed to be different. While arsenicals interact with the carbohydrate metabolism and influence membrane integrity, diamidines are thought to bind to negatively charged molecules such as nucleic acids and membrane phospholipids (Barrett and Fairlamb, 1999). Cross-resistance was therefore attributed to the loss of the P2 transporter (Carter et al., 1995; de Koning, 2001a). Here, we report cross-resistance between melarsoprol and pentamidine in laboratory-selected *T.b. rhodesiense* populations. The observed resistance factors were clearly higher than attributable to loss of the P2 transporter alone, for genetic disruption of *TbAT1* resulted in only two- to three-fold resistance to melarsoprol and pentamidine (Matovu et al., 2003). Moreover, the cross-resistance

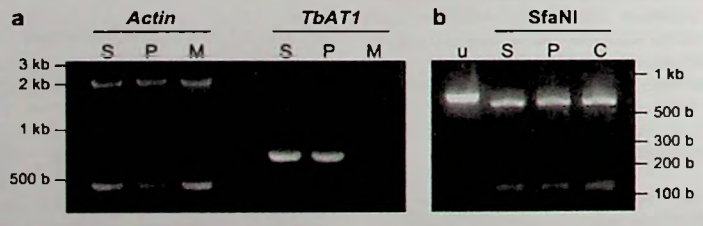

Fig. 1. Probing for *TbAT1* alleles by PCR. (a) *TbAT1* was amplified from sensitive parental (S) and pentamidine-selected (P) trypanosomes. No product was obtained from the melarsoprol-selected cells (M). Actin served as a positive control. (b) Restriction digest of the *TbAT1* PCR products with the endonuclease SfaNI. Parental and pentamidine-selected trypanosomes both exhibited the pattern typical of *TbAT1*' alleles (C, *TbAT1*' control; u, undigested).

observed here was not perfectly reciprocal. While the melarsoprol-selected population was as resistant to pentamidine (resistance factor: 174.1) as the pentamidine-selected population (resistance factor: 164.7), the pentamidine-selected population was less resistant to melarsoprol (resistance factor: 12.7) than the melarsoprol-selected one (resistance factor: 24.9). The high levels of resistance obtained here indicate accumulation of several mutations during the course of selection. Some mutations may be shared between the melarsoprol- and pentamidine-selected populations, others not, as indicated by the asymmetry of the drug sensitivity phenotypes. The melarsoprol-selected population lacked the *TbAT1* gene. There is precedence for loss of *TbAT1* both from the field (Matovu et al., 2001) and from the laboratory (Lanteri et al., 2006). The pentamidine-selected population was identical to the parental ST1B 900 in the diagnostic PCR test which is based on known resistance mutations in *TbAT1*. However, other mutations affecting TbAT1 function cannot be excluded at this point.

Melarsoprol- and pentamidine-selected populations both showed only intermediate cross-resistance to furamidine (resistance factor: 12.4). Assuming that the high-level resistance of the melarsoprol-selected population to pentamidine is caused by concomitant loss of TbAT1 and HAPT, the low cross-resistance to furamidine would indicate that furamidine uptake is HAPT-independent.

The findings of this study may be summarised as follows: (i) High-level drug resistance was obtained in vitro, with asymmetrical cross-resistance between melarsoprol and pentamidine; (ii) The melarsoprol-selected population has lost the *TbAT1* gene and therefore the P2 transporter and showed a strong resistance to pentamidine whereas the pentamidine-selected population still contained the *TbAT1* gene and showed only an intermediate resistance to melarsoprol; (iii) Both resistant populations showed an intermediate resistance to furamidine; and (iv) in contrast to the furamidine-selected trypanosomes (DB75^{R}-CL1) by Lanteri et al. (2006), the obtained resistance phenotype was manifest in vivo as well as in vitro over long periods of time.

In conclusion, drug-resistant populations represent valuable tools in drug screening and development. Understanding the mechanism underlying drug resistance is important for gaining new insights that may help to evaluate new drug targets that are needed for rational drug design. The presence of cross-resistance would indicate that the drugs tested have the same target or use the same transporter. The primary aim of this work was to develop populations of trypanosomes showing high resistance to compounds already used in the field, to assess their possible cross-resistance with new compounds. Such a therapeutic sieve based on cross-resistance phenotypes was already proposed by Ehrlich (1907) to facilitate lead development. Testing cross-resistance of drug candidates may lead to better evaluation of lead candidates and therefore may save money and time within the drug development process.

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Chapter 3.0c

Loss of the High-Affinity Pentamidine Transporter Is Responsible for High Levels of Cross-Resistance between Arsenical and Diamidine Drugs in African Trypanosomes.

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I performed the western blot and contributed Figure 9.

Supplemental Material can be found at:
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Loss of the High-Affinity Pentamidine Transporter Is Responsible for High Levels of Cross-Resistance between Arsenical and Diamidine Drugs in African Trypanosomes[S]

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ABSTRACT

Treatment of many infectious diseases is under threat from drug resistance. Understanding the mechanisms of resistance is as high a priority as the development of new drugs. We have investigated the basis for cross-resistance between the diamidine and melaminophenyl arsenical classes of drugs in African trypanosomes. We induced high levels of pentamidine resistance in a line without the *tbat1* gene that encodes the P2 transporter previously implicated in drug uptake. We isolated independent clones that displayed very considerable cross-resistance with melarsen oxide but not phenylarsine oxide and reduced uptake of [^{3}H]pentamidine. In particular, the high-affinity pentamidine transport (HAPT1) activity was absent in the pentamidine-adapted lines, whereas the low affinity pentamidine transport (LAPT1) activity was unchanged. The parental *tbat1*$^{-/-}$ line was sensitive to lysis by melarsen oxide, and this process was inhibited by low concentrations of pentamidine, indicating the involvement of HAPT1. This pentamidine-inhibitable lysis was absent in the adapted line KO-B48. Likewise, uptake of the fluorescent diamidine 4′,6-diamidino-2-phenylindole dihydrochloride was much delayed in live KO-B48 cells and insensitive to competition with up to 10 μM pentamidine. No overexpression of the *Trypanosoma brucei brucei* ATP-binding cassette transporter TbMRPA could be detected in KO-B48. We also show that a laboratory line of *Trypanosoma brucei gambiense*, adapted to high levels of resistance for the melaminophenyl arsenical drug melarsamine hydrochloride (Cymelarsan), had similarly lost TbAT1 and HAPT1 activity while retaining LAPT1 activity. It seems therefore that selection for resistance to either pentamidine or arsenical drugs can result in a similar phenotype of reduced drug accumulation, explaining the occurrence of cross-resistance.

Trypanosoma brucei subspp. are protozoan parasites that cause human African trypanosomiasis (i.e., sleeping sickness) and the corresponding veterinary condition in livestock. Treatment of both the human and livestock diseases depends on a very small set of mostly very old drugs. The first-line treatment for the late stage of both West African and East African human African trypanosomiasis is melarsoprol, an organoarsenic compound of the melaminophenyl arsenical class, introduced in 1949 (Jannin and Cattand, 2004). A similar but water-soluble melaminophenyl arsenical, melarsamine hydrochloride (Cymelarsan), is increasingly used for animal trypanosomiasis. Early-stage West African sleeping sickness is routinely treated with the diamidine drug pentamidine, introduced in 1937 (Jannin and Cattand, 2004). The corresponding widely used veterinary diamidine is diminazene aceturate (Berenil). The only new trypanocide to be developed in recent decades, DB75, is also a diamidine and currently in clinical trials as an orally available prodrug.

It has been known for decades that cross-resistance between melaminophenyl arsenicals and diamidine drugs sometimes occurs (Fulton and Grant, 1955; Williamson and Rollo, 1959), but cross-resistance patterns can be unpredictable (Williamson, 1970). Kaminsky and Maser (2000) distin-

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ABBREVIATIONS: LAPT1, low-affinity pentamidine transporter; HAPT1, high-affinity pentamidine transporter; ABC, ATP-binding cassette transporter; TbAT1, *Trypanosoma brucei* adenosine transporter 1; MRPA, multidrug resistance protein A; TbMRPA, *Trypanosoma brucei* multidrug resistance protein A; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DIC, differential interference contrast; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; RT, room temperature; PFR, paraflagellar rod protein; WT, wild-type.

guished six distinct resistance profiles in *Trypanosoma brucei brucei* laboratory strains and field isolates, presumably an indication that multiple factors determine (the level of) resistance to the various drugs. They also noted that some resistance profiles seem to be associated with either field or laboratory-induced resistance.

Some progress has been made in understanding drug resistance mechanisms in trypanosomes. The important role of the *T. brucei* AT1/P2 purine transporter in the accumulation of melaminophenyl arsenical compounds and diamidines is now well understood (Carter and Fairlamb, 1993; Maser et al., 1999; De Koning, 2001a; Delespaux and De Koning, 2007). The loss of TbAT1/P2 alone was shown to be sufficient for high levels of diminazene aceturate resistance in *T. brucei brucei* and *Trypanosoma evansi* (De Koning et al., 2004; Witola et al., 2004). However, the deletion of the *TbaT1* gene produced only minor loss of sensitivity to pentamidine and melaminophenyl arsenicals (Matovu et al., 2003). Thus, the action of some diamidines relies (almost) exclusively on TbAT1/P2 for access to intracellular targets, whereas other diamidines, as well as melaminophenyl arsenicals, have at least one additional route of entry.

We have shown previously that adenosine-insensitive uptake of [^{3}H]pentamidine in *T. brucei brucei* is mediated by two discrete transport activities: a low-affinity pentamidine transporter [LAPT1 (De Koning and Jarvis, 2001)] and a high-affinity pentamidine transporter [HAPT1 (De Koning, 2001b)]. Although this seems to explain why clinical pentamidine resistance has not established itself, despite the many decades of intensive use (for review, see Bray et al., 2003), it was not clear whether high levels of pentamidine resistance might ensue with the consecutive loss of TbAT1/P2 and either HAPT1 or LAPT1. Nor is it yet clear why the *tbat1*$^{-/-}$ line did not display an appreciable level of resistance to melaminophenyl arsenical drugs (Matovu et al., 2003). It has been argued that higher levels of arsenical resistance in trypanosomes could be the result of the (over)-expression of an ABC-type transporter of the multidrug resistance type, TbMRPA. Overexpression of this transporter in *T. brucei brucei* does induce 10-fold resistance to melarsoprol in vitro (Shahi et al., 2002). Arsenical resistance attributed to loss of TbAT1/P2 and overexpression of TbMRPA were shown to be additive (Lüscher et al., 2006). However, TbMRPA overexpression did not lead to arsenical resistance in vivo, and TbMRPA overexpression could not be demonstrated in melarsoprol-resistant trypanosome isolates from patients with sleeping sickness (Alibu et al., 2006).

In the current article, we address the outstanding questions concerning diamidine and arsenical resistance using *Trypanosoma brucei* lines adapted to high levels of pentamidine and cross-resistant to melaminophenyl arsenicals. We now provide a model for the biochemical basis of cross-resistance between melaminophenyl arsenicals and the diamidines. The model predicts that HAPT1, together with TbAT1, may be a key determinant for arsenical resistance in African trypanosomes, providing a hypothesis that should now be validated on clinical isolates from patients refractory to melarsoprol.

Materials and Methods

Trypanosomes and Cultures. Growth of bloodstream trypanosomes in culture was performed using HMI-9 medium (BioSera Ltd., East Sussex, UK) (Hirumi and Hirumi, 1989) supplemented with 2 mM β-mercaptoethanol and 10% fetal bovine serum at 37°C, in a 5% CO_2 atmosphere. For transport assays, trypanosomes were grown in adult female Wistar rats and isolated from infected blood as described previously (De Koning and Jarvis, 1997). The cloned *Trypanosoma brucei gambiense* type 2 stock STIB 386 and the derived melarsamine hydrochloride-resistant clone 386MelCy^R (hereafter called 386Ms and 386Mr) were a kind gift of Professor C.M.R. Turner (University of Glasgow). The adaptation of 386Ms to high levels of melarsamine hydrochloride has been described elsewhere (Scott et al., 1996). The *T. brucei brucei* clone s427 (MiTat 1.2/221) was used to derive the *tbat1*$^{-/-}$ line (Matovu et al., 2003) and the pentamidine-adapted clonal line WT-D24. *tbat1*$^{-/-}$ was also adapted to higher levels of pentamidine resistance, generating the clonal lines KO-B6, KO-D6, KO-B48, and KO-D48. Clonal populations were generated by limiting dilution. Cell densities were assessed using a hemocytometer and phase-contrast microscope. The New York line of *T. brucei brucei* (Wirtz et al., 1999) and the derived MRPA overexpressing line NY-mrpa have been described previously (Lüscher et al., 2006). Overexpression was induced (NY-mrpa$^+$) or repressed (NY-mrpa$^-$) by the inclusion or omission, respectively, of 1 μg/ml tetracycline in the culture medium.

Transport Assays. Transport assays were performed exactly as described previously (Wallace et al., 2002). In brief, trypanosomes were isolated from blood taken from infected Wistar rats by DEAE-52 (Whatman, Maidstone, UK) anion exchange chromatography, washed into assay buffer, and resuspended at ~2×10^7 cells/ml at room temperature. In a minority of cases, trypanosomes for transport assays were cultured in complete HMI-9 medium. Aliquots (100 μl) of bloodstream trypanosomes in assay buffer were mixed with an equal volume of buffer containing radiolabel and sometimes inhibitor, at twice the final concentration, initiating transport. Transport was terminated by the addition of 1 ml of ice-cold buffer containing unlabeled permeant at a saturating concentration (2 mM for pentamidine) followed by rapid centrifugation through an oil layer, separating cells from radiolabel not internalized. Transport values are expressed as picomoles per 10^7 cells per second.

Drug Sensitivity Assays. Drug sensitivity was determined using the dye resazurin (Alamar blue) exactly as described previously (Wallace et al., 2002) in a protocol adapted from Raz et al. (1997). In brief, drugs were serially diluted in 100 μl of complete HMI-9 media across a 96-well microtiter plate. Unless limited by solubility, the top drug concentration used was 1 mM. Cultures of bloodstream-form trypanosomes were grown to a maximum density of 2×10^6 cells/ml and diluted to 2×10^5 cells/ml with complete HMI-9 medium, of which 100 μl was added to all wells. Plates were then incubated for 48 h at 37°C in a 5% CO_2 atmosphere, before the addition of 20 μl of 5 mM resazurin (Sigma, St. Louis, MO) solution in PBS, pH 7.4. Plates were incubated for an additional 24 h at 37°C, before fluorescence was measured using a LS 55 luminescence spectrometer (λ_{ex} = 530 nm, λ_{em} = 590 nm; PerkinElmer Life and Analytical Sciences, Boston, MA).

Fluorescence Microscopy. *T. brucei* were pelleted (600*g*, 10 min at room temperature) before being resuspended in fresh HMI-9 media containing 10 μM concentrations of stilbamidine, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), DB75, or Hoechst 33342 solution at room temperature and assessed by fluorescence microscopy. Glass slides of the culture were prepared and examined using differential interference contrast (DIC) and fluorescence microscopy (λ_{ex} = 365 nm; λ_{em} = 445 nm) on an Axioplan 2 imaging microscope (Carl Zeiss GmbH, Jena, Germany) using Volocity v. 3.7 software (Improvision, Coventry, UK). Where necessary, parasites were fixed by incubating in PBS containing 2.5% glutaraldehyde for 20 min. Cells were then washed in 0.05 M glycine in PBS before being mounted on slides.

Lysis Assays. The lysis assays were performed essentially as described by Fairlamb et al. (1992), using bloodstream trypanosomes of *tbat1*$^{-/-}$ and KO-B48 grown in vitro. They were washed and

resuspended in fresh HMI-9 medium with 10% fetal calf serum at ~2×10^7 cells/ml, which was transferred to 1-ml cuvettes. Absorption was monitored, at 750 nm, at 30-s intervals. Melarsen oxide or phenylarsine oxide were added after 15 min to allow the recording of a short baseline. Up to 16 cultures were monitored simultaneously, using two HP8453 spectrophotometers (Hewlett-Packard), in the presence or absence of low concentrations of pentamidine, which by themselves did not affect cell viability or motility over the course of the experiment.

Preparation of Plasma Membrane Protein-Enriched Fractions. *tbat1*$^{-/-}$ and KO-B48 plasma membrane-enriched samples were generated by a modification of the procedure described by Voorheis et al. (1979). In brief, bloodstream-form trypanosomes were cultured in vivo in adult female Wistar rats infected by i.p. injection. Blood was collected at peak parasitemia under terminal anesthesia, and parasites were isolated as for transport assays. Cells were then osmotically stressed by addition of water (at 4°C), and swelling was monitored by phase-contrast microscopy until a spherical morphology had been achieved. Cells were then homogenized in an AO Cell Disruptor (Stansted Fluid Power, Stansted, UK), in the presence of protease inhibitors. The homogenate was then returned to a normal osmotic potential through the addition of 3 M KCl to a final concentration of 140 μM, before pelleting the cells. Cells were then treated with 240 units of DNase in TES buffer (20 mM TES, 150 mM KCl, 5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol, pH 7.4), for 5 min at 20°C. The reaction was terminated by the addition of 5 volumes of TES buffer (20 mM TES, 150 mM KCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol, pH 7.4). Cells were pelleted and then re-suspended in 40% sucrose before being layered on a linear 40 to 60% sucrose gradient (in TES buffer). Samples were then centrifuged for 3 h at 70,000*g* on a SW28 rotor (Beckman Coulter, Fullerton, CA). The most prominent dense white band, corresponding to the plasma membrane fraction, was isolated and washed twice in TES buffer to remove sucrose, before being aliquoted and stored in TES buffer at −80°C. All procedures were performed at 4°C unless otherwise stated.

Western Blotting. Samples for Western blotting were plasma membrane preparations (described under *Preparation of Plasma Membrane Protein-Enriched Fractions*) or whole-cell protein extracts prepared from 2×10^7 cells, washed in PBS, and flash-frozen in dry ice/ethanol. Samples solubilized in standard Laemmli buffer (60°C for 10 min) were loaded onto a 10% SDS polyacrylamide gel and run until the bromphenol blue dye front reached the end of the gel. Proteins were then transferred onto a nitrocellulose membrane (Hybond ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK) at 4°C in MeOH Tris-glycine buffer. Blots were incubated in blocking solution (TBS-Tween 20 containing 5% milk powder) for 1 h at room temperature (RT). Primary antibodies raised to MRPA, kindly provided by Christine Clayton, University of Heidelberg, were diluted 1:500 in blocking solution (TBS-Tween 20 containing 5% milk powder) and incubated with the blot for 1.5 h at RT. Unbound/excess antibodies were removed by washing in TBS-Tween 20 (2 × 10 min) and then TBS (10 min). Secondary peroxidase-conjugated anti-mouse immunoglobulin antibodies (Dako Denmark A/S, Glostrup, Denmark) were then incubated with the blot (diluted 1:1000) in blocking solution at RT for 1 h. Unbound secondary antibodies were removed by washing (as with primary antibodies). Bound antibodies were detected using the ECL Plus western blotting detection System (GE Healthcare) according to the manufacturer's instructions. For normalization, the blot was stripped and reprobed using a polyclonal antiserum against paraflagellar rod protein (PFR; diluted 1:1000) as described previously (Schlaeppi et al., 1989) and a secondary peroxidase-conjugated anti-rat immunoglobulin antibody (diluted 1:10,000) (Dako Denmark A/S).

Infectivity of Pentamidine-Adapted *T. brucei brucei* Lines in Mice. Groups of three to five ICR mice (Harlan UK Ltd., Bicester, Oxon, UK) were infected with 10^4 trypanosomes in 200 μl of full HMI-9/fetal calf serum culture medium by i.p. injection. In some cases, the mice had been immunosuppressed by treatment with cyclophosphamide (200 mg/kg cyclophosphamide in water, injected i.p.) 24 h before infection. Parasitemia was estimated daily by the rapid "matching" method (Herbert and Lumsden, 1976) from examinations of wet blood films on microscope slides, collected by tail prick, using a phase-contrast microscope. Mice reaching a parasitemia of antilog 8.4 (2.5×10^8 cells/ml blood) or over were euthanized by inhalation of a rising concentration of CO_2.

Results

Induction of High-Level Pentamidine Resistance in *T. brucei brucei*. To maximize the likelihood of the successful generation of drug-resistant lines, we applied selection pressure to four separate cultures from each of the two parental strains, s427 (WT) and the isogenic clone *tbat1*$^{-/-}$, the latter of which has been derived from the former by deletion of both copies of the *TbAT1* gene (Matovu et al., 2003). From these 8 cultures initially established, three (WT-D, KO-B, and KO-D) went on to develop a stable drug resistance phenotype after several months of selection in increasing pentamidine concentrations. Two clones were derived from each of these lines: one that tolerated intermediate levels of pentamidine (clones WT-D3, KO-B6, and KO-D6) and one that tolerated high levels of pentamidine (clones WT-D24, KO-B48, and KO-D48), compared with the parental strains (Fig. 1A). The strains displaying the highest levels of resistance were able to survive in pentamidine concentrations 80-fold higher than those tolerated by their parental strain. It was also observed that resistance acquisition was nonlinear over time and seemed to correlate best to an exponential model, with correlation coefficients between 0.87 and 0.93 (plots not shown). The wild-type strain, in particular, took many passages to acquire any pentamidine resistance but subsequently adapted to very significant levels of resistance in two discrete steps.

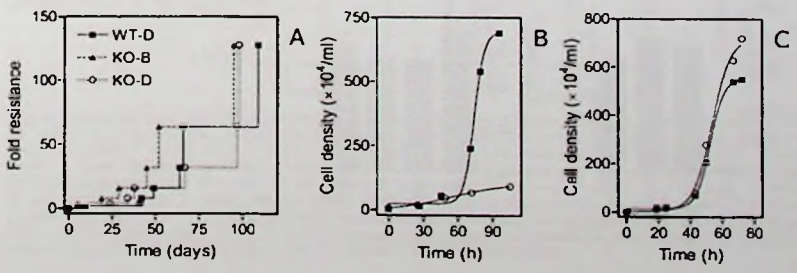

Fig. 1. Induction of pentamidine resistance in *T. brucei brucei*. A, development of resistance over time in three independent clones. B and C, growth of KO-B48 line in the presence (○) or absence (■) of 80 nM pentamidine in the growth medium. Frame B, shortly after passage to 80 nM pentamidine; frame C, after 2 month in 80 nM pentamidine.

During each round of selection, it was observed that the initial acquisition of resistance in the presence of drug was associated with a reduced growth phenotype (Fig. 1B), in terms of both doubling time and maximum cell density. For all the lines eventually selected, this was reversed to a parental growth phenotype by maintaining the same selection pressure for an additional period of time (Fig. 1C). Once the resistance phenotype was well established, removal of pentamidine from the media had no effect on growth, and the resistance phenotype was stable for at least 3 months in the absence of drug pressure.

Cross-Resistance Profile of KO-B48 and KO-D48. We have previously reported the cross-resistance phenotype of the *tbat1*$^{-/-}$ strain (Matovu et al., 2003), which displayed high levels of resistance to diminazene but only minor loss of sensitivity to pentamidine and the melaminophenyl arsenical drugs melarsoprol, melarsen oxide, and melarsamine hydrochloride. Using the same protocol based on the reduction of the dye Alamar blue (resazurin), we have repeated these experiments with WT, *tbat1*$^{-/-}$, KO-B48, and KO-D48 in parallel. The results are presented as a series of bar graphs in Fig. 2. The phenotypes of KO-B48 and KO-D48 were very similar. Compared with the WT strain, a large increase in pentamidine resistance was observed, reaching an average 130- and 82-fold resistance in KO-B48 and KO-D48, respectively ($n = 8$; Fig. 3). Resistance to melarsen oxide was also markedly increased in KO-B48 and KO-D48 (15.4- and 10.7-fold, respectively, relative to WT; Fig. 2B). Sensitivity to propamidine and stilbamidine, already reduced in the KO strain, was modestly decreased further in KO-B48 and KO-D48 (Fig. 2, C and D), whereas resistance to diminazene aceturate was not further increased in the pentamidine resistant lines (Fig. 2E). Sensitivity to phenylarsine oxide, which freely diffuses across membranes, was identical in all the lines (Fig. 2E), showing that the resistance phenotype was most likely associated with changes in drug uptake. A table with exact sensitivities of all four trypanosome lines to all six drugs is included as on-line only Supplementary Data.

Pentamidine Transport in Pentamidine-Resistant Trypanosome Lines. Transport of 0.5 μM [^{3}H]pentamidine was drastically reduced in both KO-B48 and KO-D48, compared with the parental *tbat1*$^{-/-}$ line (Fig. 4A,B), by 74 and 68%, respectively. To exclude the possibility that culture conditions had somehow played a role in the reduced uptake rates, the experiment was repeated with *tbat1*$^{-/-}$ and KO-B48 cells grown in Wistar rats (Fig. 4C). The result clearly demonstrates that pentamidine transport in the adapted lines is severely impaired yet remains saturable (Figs. 4C and 5). We also confirmed that transport in KO-B48 was not generally impaired, using [^{3}H]2-deoxyglucose. Uptake of [^{3}H]2-deoxyglucose by KO and KO-B48 cells was determined in three independent experiments, each performed in triplicate, and found to be linear over 30 min, completely saturated by 10 mM 2-deoxyglucose, and not significantly different between the two trypanosome lines (Supplementary Data Fig. 1).

Pentamidine transport in the *tbat1*$^{-/-}$ strain is mediated by two transporters, HAPT1 and LAPT1 (Matovu et al., 2003). To determine whether one or both of these transporters were affected in the highly resistant line KO-B48, a series of transport assays was performed at 15 nM [^{3}H]pentamidine. This very low concentration of radiolabel is necessary to

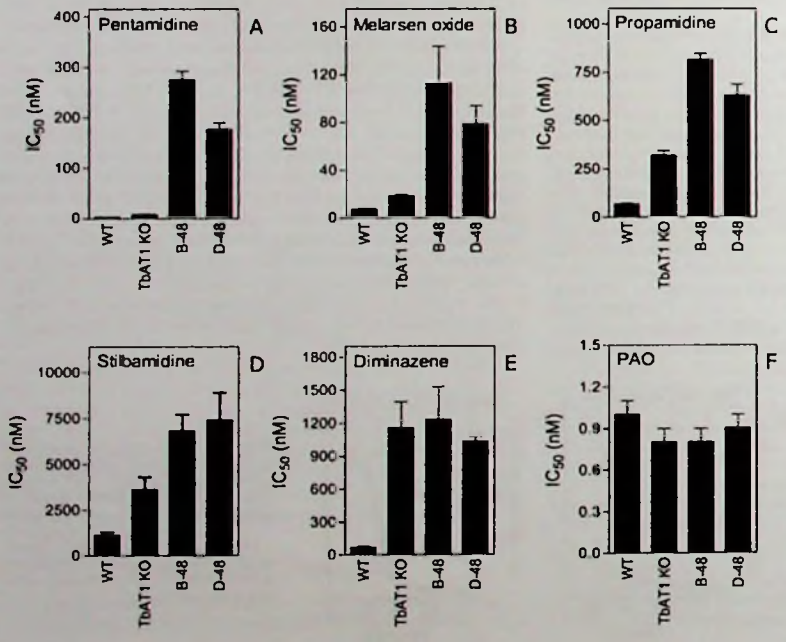

Fig. 2. Drug sensitivity profile of four *T. brucei brucei* clones. A, pentamidine. B, melarsen oxide. C, propamidine. D, stilbamidine. E, diminazene aceturate. F, phenylarsine oxide (PAO). Error bars indicate S.E. All values are the average of between 3 and 11 determinations. A table with the exact values and number of replicates is available as on-line only supplementary material.

avoid saturating HAPT1 and to obtain a biphasic inhibition curve showing both the high-affinity (but low-capacity) HAPT1 activity and the low-affinity (but high-capacity) LAPT1 activity in the WT s427 and *tbat1*$^{-/-}$ strains (De Koning, 2001b; Matovu et al., 2003). The presence of a high-affinity pentamidine transport activity could not be confirmed in KO-B48: it was either absent altogether or its transport capacity was very significantly reduced (Fig. 5A), although pentamidine transport was clearly biphasic in the control WT strain (Fig. 5B). In contrast, the low-affinity transporter LAPT1 seemed completely unchanged compared with parental strains (Fig. 5, A and C): K_m and V_{max} values were identical to those previously reported for the parental line s427 (Table 1).

Arsenical-Induced Lysis in *tbat1*$^{-/-}$ Is Mediated by HAPT1 and Absent in KO-B48. Lysis of trypanosomes, induced by arsenical drugs, can be monitored spectroscopically at 750 nm (Fairlamb et al., 1992) and was used to demonstrate that melaminophenyl arsenicals enter *T. brucei brucei* principally through P2 (Carter and Fairlamb, 1993). We have previously used this technique to show that in *tbat1*$^{-/-}$ cells, a secondary, slower phase of melarsen oxide-induced lysis can be discerned and displays the pharmacological profile of HAPT1 (Matovu et al., 2003). The results depicted in Fig. 6A confirm this observation. Although melaminophenyl arsenicals act more slowly on *tbat1*$^{-/-}$ than on WT cells (Matovu et al., 2003), 10 μM melarsen oxide induced cell lysis in *tbat1*$^{-/-}$ cells in approximately 50 min (trace *a*), but coadministration of as little as 1 μM pentamidine with 10 μM melarsen oxide substantially delayed cell lysis (trace *b*). In contrast, the same concentration of pentamidine had no influence on lysis induced by phenylarsine oxide (traces *c* and *d*), which rapidly diffuses across membranes (Carter and Fairlamb, 1993).

In KO-B48 cells, melarsen oxide-induced lysis is already much delayed compared with *tbat1*$^{-/-}$ cells (trace *a*), and 1 μM pentamidine has no further effect (trace *b*), demonstrating the absence of HAPT1 activity. Again, coincubation with 1 μM pentamidine had no effect on lysis induced by phenylarsine oxide (traces *c* and *d*), and the timing of phenylarsine-induced lysis was identical in both strains.

Assessment of Pentamidine Transporters Using Fluorescent Microscopy. The rate of entry of fluorescent diamidines, monitored by fluorescence-activated cell sorting or fluorescence microscopy, can be used as a probe to investigate changes in membrane permeability and cross-resistance patterns (Frommel and Balber, 1987; Stewart et al., 2005). Like Frommel and Balber (1987), we used 4′,6-diamidino-2-phenylindole dihydrochloride as a fluorescent substitute for pentamidine. This was possible given the fact that DAPI displays considerable affinity for all three pentamidine transporters. K_i values are 0.67 ± 0.12 μM (TbAT1/P2, n = 3), 26 ± 6 μM (HAPT1, n = 3), and 20 ± 6 μM (LAPT1, n = 5) (data not shown). The highest affinity was for TbAT1/P2; nonetheless, this transporter did not primarily mediate DAPI transport. Figure 7 shows that the rate of development of fluorescence was not clearly different in live long-slender WT s427 or *tbat1*$^{-/-}$ cells. By 5 min, fluorescence was clearly observed, and by 10 min, staining of both nucleus and kinetoplast was very intensive. In contrast, KO-B48 cells showed only faint fluorescence in both organelles after 20 min. Even after 60 min, fluorescence was much less than that observed in the

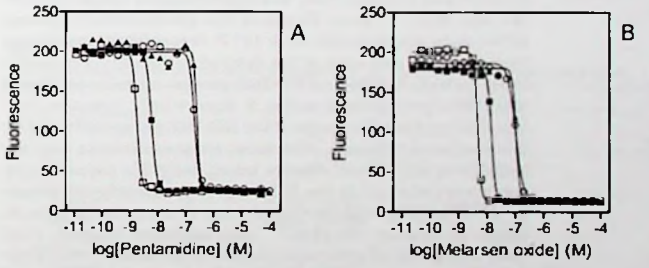

Fig. 3. Sensitivity of four *T. brucei brucei* clones to pentamidine (A) and melarsen oxide (B). Lines used were WT (□), KO, (■), KO-B48 (▲), and KO-D48 (○). The data shown is a representative experiment using the Alamar blue method as described under *Materials and Methods* section. IC_{50} values were calculated by nonlinear regression. Fluorescence is given in arbitrary units on a scale of 0 to 1000. The lowest concentration data point is the "no drug" control.

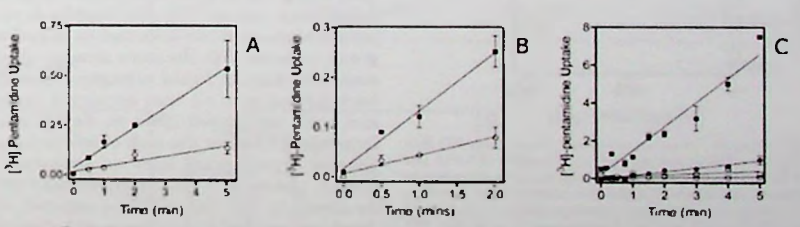

Fig. 4. Transport of 0.5 μM [^{3}H]pentamidine in resistant trypanosome lines. A, uptake of pentamidine by KO-B48 (□) or the parental *tbat1*$^{-/-}$ strain (■) grown in culture in the absence of pentamidine pressure and the two strains were assayed simultaneously. Lines were calculated by linear regression. B48, r^2 = 0.89, slope = 0.027 ± 0.005; *tbat1*$^{-/-}$, r^2 = 0.99, slope = 0.10 ± 0.006. B, uptake of pentamidine by KO-D48 (○) or the parental *tbat1*$^{-/-}$ strain (●) grown in culture in the absence of pentamidine pressure and the two strains were assayed simultaneously. Lines were calculated by linear regression. D48, r^2 = 0.97, slope = 0.038 ± 0.005; *tbat1*$^{-/-}$, r^2 = 0.99, slope = 0.17 ± 0.01. C, uptake of [^{3}H]pentamidine by KO-B48 (□, ●) or the parental *tbat1*$^{-/-}$ strain (■, ○) grown in Wistar rats, in the presence (open symbols) or absence (filled symbols) of 1 mM nonlabeled pentamidine. Lines were calculated by linear regression. Values in the absence of pentamidine were as follows: B48, r^2 = 0.97, slope = 0.0032 ± 0.0001; *tbat*$^{-/-}$, r^2 = 0.94, slope = 0.021 ± 0.002. Pentamidine uptake is expressed as picomoles per 10^7 cells. Symbols represent average of triplicate determinations; error bars are S.E., when not shown fall within the symbol.

other cell lines at 10 min (Fig. 7). Care was taken to exclude any dead cells from the observations, as they accumulated DAPI very rapidly, presumably as a result of loss of membrane integrity.

It thus seems that the rate of DAPI uptake is much reduced in KO-B48 cells, relative to the parental line *tbat1*$^{-/-}$. To assess whether this was caused by the loss of HAPT1 or LAPT1, the development of fluorescence was again monitored in live KO-B48 cells incubated with DAPI alone or in the presence of either 10 μM pentamidine (saturates HAPT1)

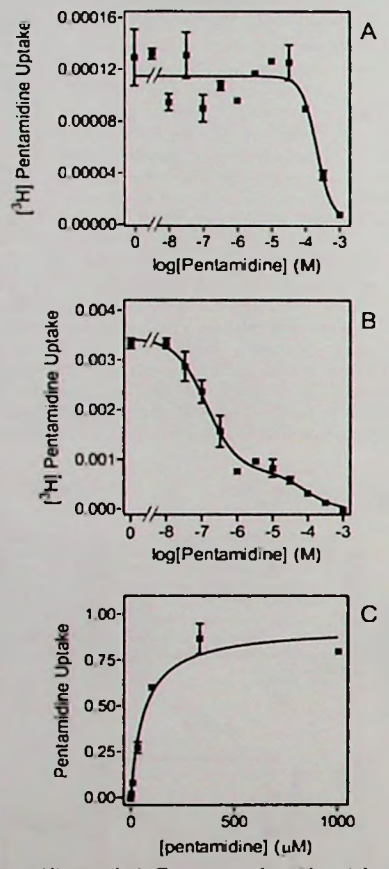

Fig. 5. Pentamidine uptake in *Trypanosoma brucei brucei* clone KO-B48 is mediated only by a low-affinity transporter. A, transport of 0.015 μM [^{3}H]pentamidine in KO-B48 over 2.5 min was assayed in the presence or absence of increasing concentrations of unlabeled pentamidine. The data were fitted to a sigmoid curve using nonlinear regression. B, uptake of 0.01 μM [^{3}H]pentamidine over 60 s in LS blood-stream forms of s427 WT was assessed in the presence of a constant concentration of 1 mM adenosine and a variable concentration of unlabeled pentamidine. The data were fitted to a "two-site competition" equation in the GraphPad Prism 4.0 software package. IC_{50} values were 0.13 and 85 μM for the high- and low-affinity components, respectively. C, conversion of the inhibition data in frame A to a Michaelis-Menten plot. All data shown are the average of triplicate determinations; error bars are S.E. Pentamidine uptake is expressed as picomoles per 10^7 cells per second.

or 1 mM pentamidine (saturates both transporters). The results in Fig. 8A show accumulation in live cells at 25, 45, and 60 min of incubation with the fluorophore. It is clear that the lower concentration of pentamidine does not delay the rate of DAPI accumulation compared with the no-drug control, whereas 1 mM pentamidine practically abolishes DAPI fluorescence over 60 min. The P-glycoprotein inhibitor verapamil (50 μM) did not affect the accumulation rate of DAPI (Fig. 8A). Using live, motile trypanosomes does not allow the overlay of DIC and fluorescent images and causes imperfect focus on the fluorescence micrographs. At the termination of the experiment, at 60 min, cells were therefore fixed with glutaraldehyde. The higher quality DIC, fluorescence, and overlay images shown in Fig. 8B confirm the conclusions of the timed/real time experiment depicted in Fig. 8A.

The Drug Efflux Transporter TbMRPA Is Not Overexpressed in KO B-48. To verify whether the cross-resistance phenotype of KO B-48 can be partly explained through the elevated expression of TbMRPA, an ABC transporter of the multidrug resistance (mdr) family, plasma membrane-enriched extracts of *tbat1*$^{-/-}$ and KO B-48 were run on one-dimensional SDS polyacrylamide gels and transferred to nitrocellulose for Western blotting with anti-TbMRPA antiserum. Protein preparations from tetracycline-induced and noninduced NY-mrpa cells and parental NY trypanosomes were analyzed in parallel as control cells. MRPA was clearly present in NY-mrpa$^+$ cells but could not be detected in any of the other lines (Fig. 9A). In contrast, the presence of PFR could easily be demonstrated in each of the preparations (Fig. 9B).

Infectivity of the KO-B48 and KO-D48 Lines. During in vivo culturing it was observed that infectivity of both KO-B48 and KO-D48 lines was much reduced compared with WT and *tbat1*$^{-/-}$ lines. To assess this systematically, groups of five mice were infected with 10^4 *T. brucei brucei* organisms from *tbat1*$^{-/-}$ and each of the derived pentamidine resistant lines. In both KO-B6 and KO-B48 groups, all mice developed detectable parasitemia within 3 days, which, however, rapidly disappeared. Two mice of the KO-B48 group suffered 1 or 3 subsequent relapses, otherwise, no parasitaemia was detectable in thick blood smears taken daily. No parasitaemia was observed at all in the KO-D6 group, and only one mouse in the KO-D48 group developed a brief, low parasitaemia on day 5. In contrast, the *tbat1*$^{-/-}$ line appeared far more virulent. One group of mice was infected with *tbat1*$^{-/-}$ cells from a long-term culture. All mice rapidly developed substantial parasitaemia, and two mice had to be euthanized. In the last group, infected with the same number of *tbat1*$^{-/-}$ cells from stabilates kept in liquid nitrogen and only briefly brought back into culture, all mice developed massive parasitaemia and were euthanized (four on day 4, one on day 13). To investigate whether the lack of infectivity was innate to the adapted trypanosome lines or dependent on the immune system, groups of immunocompromised mice were infected. As shown in Fig. 10A, all mice infected with *tbat1*$^{-/-}$ cells developed similar parasitaemia and were euthanized on day 7. In the KO-B48 and KO-D48 groups (Fig. 10, B and C), all mice similarly established substantial parasitaemia; in all but one mouse in the KO-B48 group, however, these were transient and the mice apparently self-cured.

An Arsenical-Resistant *T. brucei gambiense* Clone Has Lost TbAT1 and HAPT1. To assess whether loss of

HAPT1 activity is peculiar to selection with pentamidine, we also assessed [^{3}H]pentamidine transport in a *T. brucei gambiense* type 2 clone, 386Mr, adapted for high levels of melarsamine hydrochloride resistance in vivo (Scott et al., 1996). The [^{3}H]pentamidine transport profile of the parental line STIB 386Ms, conformed to the same three-transporter model as the earlier characterized *T. brucei brucei* s427 (De Koning, 2001b; Bray et al., 2003). Using fluorescence microscopy similar to the experiments shown here, it has previously been shown that 386Mr accumulates the diamidine DB99 much more slowly than the parental line (Stewart et al., 2005).

Figure 11A shows that uptake of 0.015 μM [^{3}H]pentamidine was inhibited partly by adenosine, with a K_i value of 1.8 μM, consistent with inhibition of TbAT1/P2. A second component, assessed in the presence of 1 mM adenosine, was revealed by a dose-dependent inhibition with propamidine, which inhibits HAPT1 but not LAPT1 (De Koning, 2001b), with a K_i value of 9.0 ± 3.0 μM (n = 3). The third transport activity was evident as inhibited by high concentrations of unlabeled pentamidine but not by propamidine or adenosine, consistent with LAPT1 activity. In contrast to 386Ms, transport of [^{3}H]pentamidine in 386Mr was not sensitive to either adenosine or propamidine (Fig. 11B), showing the absence of TbAT1 and HAPT1 activity in these cells.

The K_m and V_{max} values of HAPT1 and LAPT1 activities in the *T. brucei gambiense* lines are listed in Table 1 and are very similar to those determined in WT *T. brucei brucei*. Table 1 also shows that the V_{max} of LAPT is not changed in the resistant *T. brucei gambiense* line, relative to its parental line.

Discussion

In principle, pentamidine resistance in African trypanosomes could be associated with changes in the intracellular drug target, with reduced uptake of the drug or with active extrusion from the cell by an ABC-type efflux pump.

It has been shown that, in wild-type *T. brucei brucei*, pentamidine uptake is very rapid and efficient, amounting to a cytosolic concentration corresponding to at least 1 mM, if the drug were to remain unbound (Damper and Patton, 1976). Such an intracellular accumulation of the drug is liable to interfere with multiple vital processes and it has long been hypothesized that resistance would most likely be associated with a reduction in cellular levels (Wang, 1995). Indeed, different authors (Wang, 1995) have demonstrated various effects of pentamidine on cellular processes. Pentamidine resistance in *T. brucei brucei* based on acquired mutations in any one target, while impossible to rule out, would thus seem unlikely.

Another potential route to drug resistance is the expression of ABC transporters capable of actively removing the toxic compound from the cell. Strong evidence couples this mechanism to drug resistance in some protozoa, including *Plasmodium* and *Leishmania* spp. and *Entamoeba histolytica* (Légaré et al., 2001; Lopez-Camarillo et al., 2003). In *T. brucei*, three ABC transporter genes have been identified (Mäser and Kaminsky, 1998). However, in studies with the P-glycoprotein inhibitor verapamil, no evidence could be found for the involvement of such transporters in multidrug resistance in *T. brucei brucei*, either in vitro or in vivo (Kaminsky and Zweygarth, 1991). Experimental overexpression of the *T. brucei brucei* ABC transporter TbMRPA

TABLE 1
Kinetic parameters of pentamidine transporters in various cloned trypanosome lines

Trypanosome Species and Line	HAPT1			LAPT1	
	K_m	V_{max}	Propamidine K_i	K_m	V_{max}
	μM	pmol/10^7 cells/s	μM	μM	pmol/10^7 cells/s
T. brucei brucei					
s427 [a]	0.036 ± 0.006	0.0044 ± 0.0004	4.6 ± 0.7	56 ± 8	0.85 ± 0.15
tbat1 $^{-/-}$ [b]	0.029 ± 0.008		13 ± 3	50 ± 17	
KO-B48	N.D.	N.D.	N.D.	56 ± 7	0.82 ± 0.20
T. brucei gambiense					
386Ms	0.038 ± 0.004	0.0022 ± 0.0008	9.0 ± 3.0	113 ± 18	2.2 ± 0.7
386Mr	N.D.	N.D.	N.D.	68 ± 14	2.1 ± 0.1

N.D., not detectable.
[a] Values from De Koning, 2001b.
[b] IC_{50} values from Matovu et al., 2003.

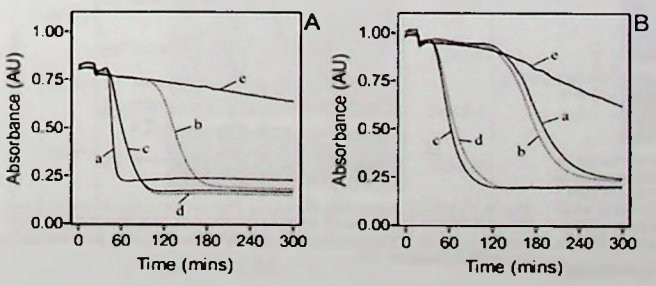

Fig. 6. Lysis of trypanosomes induced by 10 μM melarsen oxide. A, strain *tbat1* $^{-/-}$. B, strain KO-B48. Melarsen oxide (10 μM) was added after 15 min of recording, as 50 μl (200 μM) in full HMI-9 medium added to 1 × 10^7 (A) or 1.5 × 10^7 *T. brucei brucei* in 1 ml of full HMI-9 medium being monitored at 750 nm. Conditions from t = 0 min: *a*, 10 μM melarsen oxide; *b*, 10 μM melarsen oxide plus 1 μM pentamidine; *c*, 0.5 μM phenylarsine oxide; *d*, 0.5 μM phenylarsine oxide plus 1 μM pentamidine; *e*, control, no drug. Dotted lines indicate the incubations in the presence of 1 μM pentamidine.

led to substantial melarsoprol resistance in vitro (Shahi et al., 2002) but not in vivo, and TbMRPA overexpression was not observed in melarsoprol-resistant clinical isolates from a number of patients with sleeping sickness (Alibu et al., 2006).

Thus, although resistance based on target modifications or active efflux cannot be excluded, the available information is consistent with changes to drug entry being the cause of arsenical and diamidine resistance in African trypanosomes. Our findings, using pentamidine-adapted laboratory strains, are strongly in agreement with this hypothesis. We identify HAPT1 as potentially a key drug resistance determinant and for the first time provide a biochemical model for high-level arsenical-diamidine cross-resistance in African trypanosomes.

Fig. 7. Assessment of DAPI uptake using fluorescent microscopy over time. LS bloodstream forms of WT s427, *tbat1*$^{-/-}$ and KO-B48 were incubated with 10 μM DAPI and fluorescence was observed under an Axioplan 2 imaging microscope (λ_{ex} = 365 nm, λ_{em} = 445 nm) at 1000× magnification.

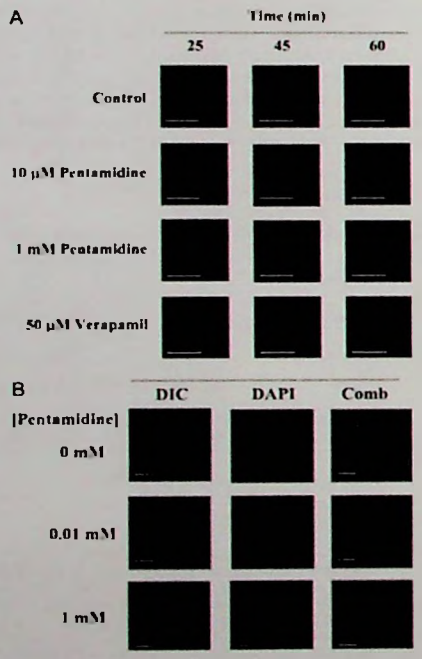

Fig. 8. DAPI fluorescence acquisition in live KO-B48 cells incubated in the absence or presence of a low (10 μM) or high (1 mM) concentration of pentamidine, or 50 μm verapamil over time (A). Separate images (DIC, DAPI, and combined overlay) from glutaraldehyde-fixed parasites after 60-min incubation with DAPI in the presence or absence of pentamidine (B). DAPI concentration was 10 μM. Magnification was 1000×. Comb, combined overlay.

High-Level Pentamidine Resistance Is Associated with Loss of HAPT1 but Not of LAPT1 Activity. Several independent lines of evidence suggested that the pentamidine-adapted lines KO-B48 and KO-D48 had lost HAPT1 rather than LAPT1 activity. Because the adaptation started with the *tbat1*$^{-/-}$ clonal line, P2 transport activity was also not present. We have shown previously that propamidine is an inhibitor of high-affinity pentamidine transport in *T. brucei* but not of the low-affinity transport component (De Koning, 2001b), and the increase in propamidine resistance is certainly consistent with loss of HAPT1 activity. Furthermore, there was no increased resistance to diminazene aceturate, which is transported only by TbAT1/P2 (De Koning et al., 2004), or to phenylarsine oxide, which diffuses across membranes.

The resistance profiles and [^{3}H]pentamidine transport rates of the two adapted lines were highly similar. These experiments measured initial rates of pentamidine uptake rather than accumulation, and the results can only be explained in terms of deficient uptake rather than active extrusion or intracellular sequestration. Unlike *T. brucei brucei* s427 and *tbat1*$^{-/-}$ (De Koning, 2001b; Matovu et al., 2003), transport of 15 nM [^{3}H]pentamidine in KO-B48 was not inhibited by submicromolar concentrations of unlabeled pentamidine, saturated only by very high levels of the substrate, and K_m and V_{max} values were identical to those reported earlier for LAPT1 (De Koning, 2001b).

In *tbat1*$^{-/-}$, lysis induced by melarsen oxide was very considerably delayed, relative to *T. brucei brucei* s427, by coadministration of as little as 1 μM pentamidine—sufficient to saturate HAPT1 but not LAPT1. Pentamidine was apparently competing with the arsenical drug for transporter-mediated import, because 1 μM of pentamidine had no effect on the rapid lysis induced by phenylarsine oxide. These experiments clearly demonstrate the uptake of melarsen oxide through HAPT1. The same process was assessed by monitor-

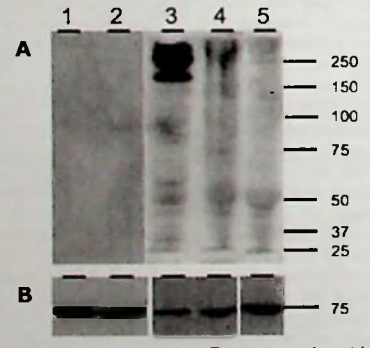

Fig. 9. TbMRPA expression in various *Trypanosoma brucei brucei* lines. Western blots with extracts of *tbat1*$^{-/-}$ (lane 1), KO-B48 (lane 2), NY-mrpa + (lane 3), NY-mrpa- (lane 4), and NY (lane 5), using anti-TbMRPA (A) or PFR antisera (B).

ing DAPI fluorescence as a marker for diamidine accumulation. Scant difference was observed between the WT and *tbat1*$^{-/-}$ lines, but the development of fluorescence was substantially delayed in KO-B48 cells (Fig. 7), indicating a loss of diamidine transport unrelated to TbAT1/P2. The remaining DAPI transport activity was saturated by 1 mM but not by 10 μM pentamidine, consistent with uptake through LAPT1 rather than HAPT1.

The Role of HAPT1 in Accumulation of Melaminophenyl Arsenicals. Since the seminal observations of Carter and Fairlamb (1993) that a *T. brucei brucei* line adapted to high levels of resistance to melaminophenyl arsenicals had lost activity of a transporter they named P2, much evidence has accumulated linking TbAT1/P2 to arsenical resistance (Carter et al., 1999; Delespaux and De Koning, 2006). Loss of P2 activity alone was not sufficient to establish substantial arsenical resistance as the *tbat1*$^{-/-}$ line was less than 3-fold resistant to melarsen oxide and melarsoprol (Matovu et al., 2003). We show here that lysis of trypanosomes induced by melaminophenyl arsenicals, but not by phenylarsine oxide, was 1) sensitive to inhibition by 1 μM pentamidine and 2) much delayed in KO-B48 cells. The level of resistance to melarsen oxide reached 15-fold in KO-B48. We conclude that high levels of arsenical resistance can be induced by selection with pentamidine and can be achieved by the sequential loss of TbAT1 and HAPT1. The example with *T. brucei gambiense* 386Mr shows that selection with melaminophenyl arsenicals can likewise lead to loss of the same two transporters, thus explaining reported incidences of pentamidine/arsenical cross-resistance (Frommel and Balber, 1987; Kaminsky and Maser, 2000). High levels of resistance could be far more easily induced when starting from a TbAT1-null background. This is potentially significant given the increasing levels of resistance to melarsoprol and diminazene aceturate in the field, believed to be at least partially linked to loss of TbAT1/P2 activity in the resistant strains (Kaminsky and Maser, 2000; De Koning et al., 2004; Delespaux and De Koning, 2006).

It must be acknowledged that our findings have been generated under laboratory conditions and that any clinical link between loss of HAPT and diamidine or melarsoprol treatment failure remains theoretical. However, the previous explanation for the observed treatment failures (loss of TbAT1/P2 only) proved unsatisfactory (Matovu et al., 2003), and we here propose an improved model that can now be tested against the situation in the field. Efforts to identify the genes encoding HAPT and LAPT are ongoing and would greatly assist the evaluation of this model against clinical isolates.

It is possible that additional mechanisms of resistance to melaminophenyl arsenicals may exist, because cross-resistance is not always observed (e.g., Fairlamb et al., 1992). However, conclusions based on a review of the literature are complicated by differences in technique (e.g., in vitro or in vivo assessment of resistance), variations in drugs tested, and other confounding factors.

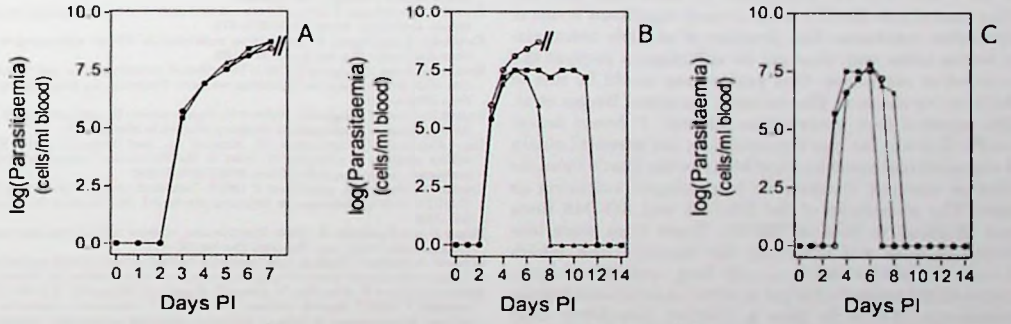

Fig. 10. Parasitaemia in *T. brucei brucei* infected mice. Progression of *T. brucei brucei* infections in immunocompromised mice after intraperitoneal inoculation with equal numbers of *tbat1*$^{-/-}$ (A), KO-B48 (B), and KO-D48 (C). Animals reaching a parasite burden of 10^9 or more were euthanized, indicated with a double bar. Each group consisted of three mice, represented by symbols ■, ▲, and ●. PI, postinfection.

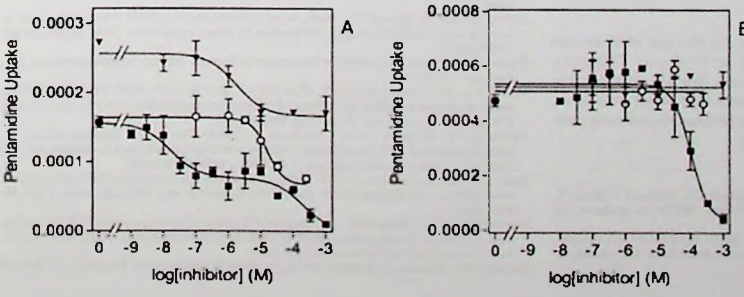

Fig. 11. Transport of 0.015 μM [^{3}H]pentamidine in *T. brucei gambiense* 386Ms (A) and 386Mr. Uptake was measured over 60 s in the presence or absence of adenosine (▼), propamidine (○), or unlabeled pentamidine (■). Incubations with propamidine or pentamidine were performed in the presence of 1 mM adenosine to saturate the TbAT1/P2 transporter. Data shown are the average of triplicate determinations; error bars are S.E. Pentamidine uptake is expressed as picomoles per 10^7 cells per second.

The Role of Drug Efflux: ABC Transporters. It has been demonstrated that overexpression of the *T. brucei brucei* ABC transporter MRPA resulted in a 10-fold resistance to melarsoprol in vitro (Shahi et al., 2002), an effect that was additive with the effect of *TbaT1* deletion (Lüscher et al., 2006). However, we found no detectable levels of TbMRPA in the two pentamidine-adapted lines. In addition, we found that the P-glycoprotein inhibitor verapamil had no effect on DAPI accumulation, and thus we have been unable to find evidence that the increased resistance phenotype is the result of increased activity of P-glycoprotein or mdr-type ABC-transporters.

Is Pentamidine Resistance Associated with Reduced Virulence? Pentamidine is still the first-line drug for early-stage gambiense sleeping sickness. Treatment failures are rare but stable over at least three decades (Pépin and Milord, 1994). In contrast, pentamidine resistance seems to emerge easily in *Leishmania* species (Papadopoulou et al., 1998), though pentamidine usage against leishmaniasis has been very much less widespread than against African trypanosomiasis. The difference can be explained in part by the involvement of an mdr-type ABC transporter in pentamidine efflux from *Leishmania* (Coelho et al., 2003) and the fact that pentamidine uptake in Leishmania is apparently mediated by a single transporter (Basselin et al., 2002; Bray et al., 2003). The presence of three pentamidine transporters in *T. brucei brucei* has been argued to be responsible for the lack of clinical resistance to pentamidine (Bray et al., 2003). However, it has been demonstrated that trypanosomes with defective *tbat1* genes can be isolated from sleeping sickness patients (Matovu et al., 2001), and we show here that the further loss of just HAPT1 leads to very significant levels of pentamidine resistance. The presence of multiple accumulation routes alone may thus not be sufficient to prevent the occurrence of resistance. One explanation would be loss of viability or virulence in the resistant parasites. Berger et al. (1995) reported that pentamidine adapted *T. brucei brucei* clone PR32.6 was far less virulent than the parental strain and excluded increased susceptibility to the host's immune system or changes attributable to prolonged culturing as factors. The phenotype of the KO-B48 and KO-D48 lines seems identical to that of PR32.6. These lines were less infective in mice and rats than the parental line, which had been cultured at least equally long, and immunosuppression of the animals did not lead to substantially higher parasitaemia. There is thus a distinct possibility that high-level pentamidine resistance is not viable in the field, and self-limiting.

Acknowledgments

We are grateful to Professor Mike Turner for the use of *T. brucei gambiense* strains 386Ms and 386Mr and to Professor Christine Clayton for the gift of anti-TbMRPA antiserum. We are particularly grateful to Professor Paul Voorheis (University of Dublin) for expert advice and assistance on the preparation of plasma membrane protein-enriched fractions for Western blotting.

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Chapter 4.0

Comparative genomics of metabolic networks of parasites and host.

I performed all the experiments. Thanks to Prof. Pascal Maeser for the help with perl scripts and data analysis.

Comparative genomics of metabolic networks of parasites and host.

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Abstract

Background

Endoparasites often lack particular metabolic pathways as compared to free-living organisms. This phenomenon comprises anabolic as well as catabolic reactions. Presumably, the corresponding enzymes were lost in adaptation to parasitism. Here we compare the predicted core metabolic graphs of obligate endoparasites and non-parasites (free living organisms and facultative parasites) in order to analyze how the parasites' metabolic networks shrunk in the course of evolution and to identify candidate drug targets.

Results

Metabolic graphs of biochemical reactions present in the presumed ancestor of parasites and non-parasites were reconstructed from the Kyoto Encyclopedia of Genes and Genomes. While the parasites' networks had fewer nodes (metabolites) and edges (reactions), other parameters such as average connectivity, network diameter and number of isolated edges were similar in parasites and non-parasites. The parasites' networks contained a higher percentage of ATP-consuming reactions. Randomly shrunk networks of the same size as the parasites' exhibited smaller diameters and more isolated edges. All the networks exhibited a scale-free frequency distribution of connectivities. Candidate drug targets were identified in the metabolic graph of *Trypanosoma brucei* and validated by reverse genetics.

Conclusions

The parasites' networks were smaller than those of the non-parasites regarding number of nodes or edges, but not network diameters. Network integrity but not scale-freeness has acted as a selective principle during the evolutionary reduction of parasite metabolism. Cobalamin-independent methionine synthase and homocysteine S-methyltransferase from *T. brucei* are

candidate drug targets since they are absent from the human proteome and essential for bloodstream-form trypanosomes at physiological levels of methionine.

Background

Unicellular endoparasites are the causative agents of a plethora of human diseases: malaria, sleeping sickness, Chagas' disease, toxoplasmosis, leishmanioses, amoebic dysentery and many more, particularly in the tropics. Obligate endoparasitism among the protozoa is of polyphyletic origin. Nevertheless, the different parasites exhibit striking similarities regarding their metabolism, which is reduced – or streamlined – compared to free-living organisms. Presumably in adaptation to life within a foreign host, the parasites have lost metabolic functions in the course of evolution [1]. For instance, all obligate endoparasitic protozoa are incapable of purine de novo synthesis; they lack the corresponding genes and import exogenous purines from their hosts to synthesize nucleic acids [2, 3]. The phenomenon also includes catabolic pathways: the intestinal parasites *Entamoeba histolytica* and *Giardia duodenalis* lack mitochondria and hence oxidative phosphorylation and respiration [4]. *Trypanosoma brucei* possess mitochondria, but these are only functional in the insect stages; the bloodstream-stages rely on substrate-level phosphorylation to generate ATP [5]; the same may apply to *Plasmodium falciparum* [6]. The trend towards metabolic simplification is also apparent from endoparasitic bacteria such as *Treponema pallidum* [7] or *Mycoplasma genitalium* [8], which lack the genes for purine and also those for pyrimidine synthesis (and the Krebs cycle as well). Thus the reduction of metabolic complexity is a convergent trait among endoparasites.

Parasitic metabolism is of interest not only to the evolutionary biologist but also to the pharmacologist. With the advent of large compound libraries and high-throughput screening facilities, the identification of suitable drug targets has become the bottleneck in antiparasitic hit discovery. Comparison of host and parasite metabolism may reveal vulnerable points for chemotherapeutic intervention, such as enzymes that are essential for the parasite but not for

the host (see for instance Plate 2 in [9]). Alternatively, prodrugs may be designed so that they are specifically activated upon metabolic conversion in the parasite [10]. Proof of principle for this strategy was obtained with purine antimetabolites targeted towards *Toxoplasma gondii* [11]. Some parasites also possess metabolic capabilities in excess of their host which, if essential, lend themselves as parasite-specific targets. Examples are folate synthesis or the DOXP-pathway for isoprenoid synthesis in the malaria parasites. Antifolates such as sulfadoxine or pyrimethamine have long been a mainstay in malaria therapy while fosmidomycin, an inhibitor of the DOXP-pathway, is a more recent addition to the antimalarial arsenal [12].

Traditionally, the metabolism of a cell has been regarded as a network of interlinked pathways [13]. More recently, representation of the metabolism as a graph, where metabolites are the nodes and enzymes are the edges (and each node appears exactly once), has provided surprising insights [14]. When the mathematical concepts of graph theory that had originally been developed for quantitative analysis of social networks were applied to metabolism, the different networks appeared to share a number of characteristics with other nets such as the world-wide web or ecological food webs. In particular, all these real-world networks exhibit a short average path-length (the 'small world') and a scale-free, i.e. power-law frequency distribution of number of edges per node [15, 16]. These characteristics are thought to result from the fact that new nodes joining the network preferentially attach to highly-linked ones ('the rich get richer' [17]). Therefore, networks are generally studied in terms of their expansion. The shrinkage of networks has only been analyzed in the context of how the targeted removal of nodes or edges leads to collapse: be it to plan epidemiological interventions that stop the spread of an infectious disease or to anticipate internet terrorists' attacks on the world-wide web. Hence there is a third reason – besides the study of

convergent evolution among parasites and the quest for new drug targets – to investigate parasite metabolism: it provides a rare opportunity to study how networks shrink in a natural way that preserves integrity and maintains functionality.

With the completion of the genome projects for a number of endoparasitic protozoa, their metabolic networks can be reconstructed *in silico* from the predicted proteomes [18-21]. Here we compare the predicted metabolic graphs of protozoan endoparasites, their human host and reference organisms, aiming to analyze how the parasite networks have shrunk and to identify new antiparasitic drug targets.

Results and Discussion

In silico reconstruction and comparison of metabolic networks from parasites

To allow for subsequent comparisons between parasite and host, we focused on reconstruction of metabolic networks on pathways that must have been present in their presumed common ancestor. These pathways comprised glycolysis, gluconeogenesis, the Krebs cycle, pentose phosphate pathway, purine and pyrimidine metabolism, and amino acid metabolism – here jointly referred to as 'core metabolism'. Educt-product pairs of the core metabolic pathways were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG [22]) *Pathway* database and used to build a reference graph of 907 nodes. Species-specific enzyme lists for the core metabolism were downloaded from KEGG *Organism* via LinkDB. The obligate endoparasites considered here for analysis of their metabolic networks were *P. falciparum, T. brucei, T. cruzi, L. major, T. parva, E. cuniculi, C. hominis* and *E. histolytica*. The following species were included for comparison: the free-living *S. pombe, S. cerevisiae, D. discoideum, D. melanogaster* and *C. elegans*, the facultative parasites *E. coli* and *C. albicans*, and the mammalian host *H. sapiens* (Table 1). The species-specific metabolic networks were built by linking the species-specific enzyme lists to educt-product pairs via the corresponding reaction numbers, obtained from KEGG *Reaction*. Two metabolites were only connected in case of carbon atom transfer between them; exchange of phosphate groups, electrons, etc. were ignored, as recommended by several authors [18, 23]. Thus the current (aka currency) metabolites such as ATP or NADH did not become hubs, rendering the networks physiologically more meaningful.

Finally, the graphs were supplemented with educt-product pairs of spontaneous, (non-enzyme-catalyzed) reactions followed by removal of all pairs of unconnected nodes (i.e. all the isolated edges). The basic graph properties were calculated with BioLayout (Table 2). The

predicted core metabolic graphs were, as expected, significantly smaller for the obligate endoparasites than for the other organisms (Figure 1a), both in average number of nodes (287 vs. 483; two-tailed Mann-Whitney test $p<0.0001$) and edges (278 vs. 539; $p<0.0001$). The facultative parasites *E. coli* and *C. albicans* grouped together with the free-living eukaryotes (Figure 1), here collectively referred to as 'non-parasites'. When the network size was measured in terms of its diameter, i.e. the longest of all direct paths between any two nodes of the graph, there was no significant difference between parasites and free-living eukaryotes (Table 2). The variance among network diameters was much larger for the parasites than for the non-parasites (Figure 1b), with values ranging from 34 for *E. histolytica* to 13 for the microsporidian *E. cuniculi*. While the nodes of the parasites' networks exhibited smaller average and maximal connectivities (Table 2; Figure 1b), there was no significant difference between the metabolic graphs of parasites and non-parasites considering the numbers of isolated edges (Table 2). Both kinds of graphs followed a scale-free, i.e. power law, frequency distribution of links per node, provided the nodes of connectivity 1 were ignored (Figure 2). The same applied to the reference network (Figure 2). This effect was caused only in part by the preceding removal of all isolated edges; also without that trimming step, the power law frequency distribution of the metabolic graphs only applied to degree 2 or more (not shown). This makes biological sense since in a perfect power law distribution the metabolic network would be dominated by dead-ended nodes of degree 1.

The present approach towards species-specific metabolic graphs was straightforward thanks to the Kyoto Encyclopedia. It depends critically on the quality of gene prediction and gene product annotation of the genome files. Thus apparent differences in network size, such as those observed between *H. sapiens* and *D. melanogaster* (Figure 1a), may partly be attributed to the better knowledge of the human proteome compared to other organisms. Nevertheless,

the clear difference in network size between parasitic and free-living species (Figure 1) is unlikely to be an artefact of gene prediction. For parasites such as *T. brucei*, *T. cruzi* and *L. major*, gene prediction is straightforward owing to the absence of introns in trypanosomatids.

How did the parasites' metabolic networks shrink?

It is safe to assume that the parasites' metabolic networks did not shrink by a random process. But which principles, respectively selective forces, governed the loss of metabolic enzymes and pathways in obligate endoparasites? Experimental graphs, generated by random removal of edges from the reference network until they reached the average size of the parasites' graphs, differed from the natural networks in that they were less coherent: the random graphs contained significantly (Kruskal-Wallis followed by Dunn's multiple comparison test, $p=0.0001$) more isolated edges than those of either parasites or non-parasites (Table 2), and even after removal of these isolated edges, nodes of degree 1 were overrepresented in the random graphs (Figure 2, intersection with ordinate). The reference, the parasite, and the non-parasite graphs, while strongly differing in number of nodes and edges, all had a diameter of around 24, which is in agreement with previous studies on *E. coli [18]*. In contrast, the randomly shrunk graphs had a significantly (Kruskal-Wallis followed by Dunn's multiple comparison test, $p=0.0024$) smaller diameter of around 15 (Table 2), reflecting the decomposition of the network into medium-sized entities.

To identify further factors that shaped core metabolism in the parasites we tested the hypothesis that the frequency distribution of the number of links per node may have exerted a selective pressure: possibly, scale-freeness had to be maintained in order to preserve the robustness of the metabolic networks. This would be in agreement with the finding that the parasite graphs' connectivities followed a power law distribution roughly in parallel to that of

the non-parasites (Figure 2). However, the randomly shrunk graphs exhibited a power-law distribution almost coincident with that of the parasites (except for the intercept with the ordinate; Figure 2). Thus scale-freeness is itself a robust trait that cannot be abolished by random deletion of edges and therefore, it cannot have acted as a selective force. Another testable hypothesis was, that during the course of evolution the parasites preferentially lost enzymes catalyzing ATP-requiring reactions for economical reasons. That would be in agreement with the fact that all obligate endoparasites have lost purine de novo synthesis, which is an energetically costly pathway (for instance, it requires more ATP than pyrimidine de novo synthesis, which was lost in fewer parasites). Contrary to expectation though, the parasites' core-metabolism contained a significantly higher percentage of ATP-requiring enzymes compared to the non-parasites (two-tailed Mann-Whitney test, $p=0.0004$). Those parasites with the smallest metabolic graphs in terms of number of nodes or edges, *E. cuniculi* and *C. hominis*, had the highest percentages of ATP-consuming reactions (Figure 1c). In fact, the percentage of ATP-consuming reactions in a given organism negatively correlated with that organism's total number of core metabolic reactions (Spearman coefficient -0.95, $p<0.0001$), indicating that ATP-consuming reactions have a higher propensity to be retained in the course of evolution. A possible interpretation could be that ATP-consuming reactions are more likely to be essential than reactions that do not involve ATP, so loss of the corresponding enzymes would be more likely to be harmful. Note that ATP is a current metabolite and did not represent a hub in the present graph analysis.

Prediction of candidate drug targets based on comparison of metabolic graphs

The 'core' metabolism as defined here does not contain the pathogen-specific pathways, such as folate de novo synthesis or isoprenoid synthesis via DOXP, that make classical antimicrobial drug targets. We nevertheless tried to identify potential drug targets based on

differences in the core metabolic graphs between parasite and host. Two complementary approaches were pursued, one focusing on the nodes, the other on the edges. First we aimed to identify metabolites that are more highly linked in the networks of parasites than in those of their host, assuming that if such metabolites can be identified, structurally related antimetabolites may be more toxic to the parasite than to the host (since the most detrimental attacks against networks are those directed towards hubs). Figure 3 shows the matrices of node connectivity distributions of *P. falciparum* and *T. brucei* versus *H. sapiens*. In all three organisms, the most highly connected nodes were pyruvate and acetyl-CoA, and the amino acids glutamate, glycine and aspartate (Figure 3), which is similar to the situation in prokaryotes [24]. Very few metabolites exhibited a higher connectivity in the networks from parasites than in the human host (lower right corners in Figure 3). The largest difference was observed for threonine in *T. brucei*, followed by diacylglyceryl-2-aminoethylphosphonate in both *T. brucei* and *P. falciparum*. Other metabolites of higher connectivity in *P. falciparum* than in *H. sapiens* included cadaverine, homocysteine, and the fatty acid precursors phosphatidylcholine, diacylglycerol and ethanolaminephosphate. The scarcity of metabolites with higher connectivity in parasites than in *H. sapiens* was somewhat surprising in spite of the difference in network size (Figure 1a), given that the two kinds of graphs differed in average connectivity by only 0.3 (Table 2). Interestingly, the randomly shrunk graphs exhibited about an order of magnitude more nodes of higher connectivity than in *H. sapiens*, than the parasites' graphs (data not shown), even though the random graphs had a smaller maximal connectivity than the parasites' graphs (Table 2). The finding that threonine was more highly linked in *T. brucei* than in *H. sapiens* (Figure 3) points towards a particular role of the amino acid in trypanosomal metabolism, which is in agreement with the fact that exogenous threonine is rapidly metabolized to acetate and subsequently used for lipid synthesis in *T. brucei* [25, 26].

Next we compiled all the reactions, respectively enzymes, which were present in parasites but not in the human host. The absence of these enzymes in *H. sapiens* was verified by blastp [27] searches against the predicted human proteome. Figure 4 summarizes the hits for the kinetoplastid parasites *T. brucei, T. cruzi, L. major* and the apicomplexan *P. falciparum*. There was no overlap between the kinetoplastids and the apicomplexan. Three of the parasite-specific enzymes were present in all three kinetoplastids: nitrilase (EC 3.5.5.1), gluconolactonase (EC 3.1.1.17), and homoserine kinase (EC 2.7.1.39). Other parasite-specific enzymes included homoserine dehydrogenase (EC 1.1.1.3) and threonine aldolase (EC 4.1.2.5) in *L. major*, arginine kinase (EC 2.7.3.3) in *T. brucei* and *T. cruzi*, and in *P. falciparum* chorismate synthase (EC 4.2.3.5) and phospholipase C (EC 3.1.4.3; Figure 4). Of particular pharmacological interest was EC 2.1.1.14, cobalamin-independent methionine synthase (5-methyltetrahydropteroyltriglutamine-homocysteine methyltransferase), due to its role in methionine recycling (see below). Cobalamin-independent methionine synthase was present in *T. brucei* and in *L. major* but not in *H. sapiens*, who possesses a cobalamin (vitamin B12)-dependent methionine synthase instead (Figure 5a). In *T. brucei*, methionine can also be recycled from homocysteine using SAM as a methyl group donor, by the enzyme homocysteine S-methyltransferase. Again, the human host uses an alternative enzyme, betaine-homocysteine S-methyltransferase, and trimethylglycine (glycine-betaine) as a methyl donor (Figure 5a).

Validation of methionine recycling enzymes as drug targets in *Trypanosoma brucei*

The pharmacological importance of methionine in African trypanosomes is not confined to the aforementioned core metabolic pathways for methionine recycling that differ from those

in the human host. Two biochemical peculiarities of *T. brucei* ssp. link to methionine metabolism: ergosterol synthesis and trypanothione synthesis (Figure 5b), and both account for proven drug targets. Trypanosomes make ergosterol and are highly susceptible to antifungal agents such as miconazole [28]. Trypanothione (bis-glutathionyl-spermidine; [29]) is essential for the defense against oxidative stress since trypanosomes lack catalase, and trypanothione metabolism is one of the prime antitrypanosomal drug targets [30]. The vulnerability of methionine metabolism is further underscored by the fact that *T. brucei* SAM synthase is not regulated, so perturbations readily propagate throuh the network [31]. Given the importance of methionine metabolism in *T. brucei*, we validated by a reverse genetic approach the candidate target enzymes 2.1.1.14 (cobalamin-independent methionine synthase) and 2.1.1.10 (homocysteine S-methyltransferase). The predicted genes in *T. brucei* were: Tb927.8.2610, whose product scored with E-values of $2.4 * 10^{-161}$ and $6.5 * 10^{-194}$ against the Pfam [32] profiles PF08267 and PF01717 for N-terminal and catalytic domain, respectively, of the cobalamin-independent methionine synthase. And Tb927.1.1270, whose product scored with an E-value of $3.5 * 10^{-8}$ against PF02574, homocysteine S-methyltransferase. Both genes were expressed in bloodstream as well as procyclic (tsetse fly midgut) stage trypanosomes as determined by reverse transcriptase PCR (data not shown). In order to investigate the function of the two genes in trypanosomes, we constructed three mutant lines based on the *T. b. brucei* bloodstream-form "single marker" (SM) strain that expresses T7 RNA polymerase and the Tet repressor [33]. In the first line both alleles of the predicted homocysteine methyltransferase gene (Tb927.1.1270) were replaced with selectable markers. The second line was stably transformed with an stem-loop RNAi construct directed against the predicted methionine synthase gene (Tb927.8.2610) under the control of Tet operators. The third line was a double mutant that expressed the methionine synthase-RNAi construct in the homocysteine methyltransferase null mutant background. Neither parental (SM) nor mutant trypanosomes

grew in the absence of methionine (Table 3), demonstrating that *T. brucei* are auxotrophic for methionine. All the lines grew in standard bloodstream-form medium, demonstrating that the two genes – alone or together – are dispensable under standard culture conditions. This is in agreement with a chromosome I-wide RNAi screen, where the presumed methyltransferase Tb927.1.1270 was not essential either [34]. However, the medium for *T. brucei* bloodstream-form cultures contains 200 μM methionine while the physiological concentration in human serum is only 30 μM methionine. We therefore measured the growth of the different *T. brucei* lines in the methionine-free medium supplemented with 30 μM methionine. Here only the parental *T. brucei* SM were proliferating (albeit not to densities as high as in 200 μM methionine), while all the mutant lines failed to grow (Table 3). Thus the entry in the TDR Targets Database [34] that Tb927.1.1270 needs to be reassessed.

Conclusions

1. Focusing on core metabolism – comprising glycolysis, gluconeogenesis, the Krebs cycle, pentose phosphate pathway, purine, pyrimidine, and amino acid metabolism – for reconstruction of metabolic graphs allowed comparative genomics between endoparasitic protozoa and non-parasitic species in an evolutionary context.

2. The reconstructed core metabolic graphs of obligate endoparasitic protozoa were significantly smaller than those of free-living or facultative parasitic species when regarding the number of predicted nodes (metabolites) or edges (reactions). However, there was no difference in network diameter. All the metabolic graphs, from parasites as well as from non-parasites, were scale-free regarding the frequency distribution of connectivities, provided that nodes of connectivity 1 were ignored.

3. Control graphs, shrunk to the average size of the parasites' networks by random deletion of edges from the reference network, also exhibited a scale-free frequency distribution of connectivities, indicating that the preservation of scale-freeness cannot have acted as a selective force on the natural networks. The randomly shrunk networks had smaller diameters and contained more isolated edges than the natural graphs.

4. The total number of reactions in a given organism negatively correlated with the percentage of ATP-consuming reactions, indicating that ATP-consuming reactions, respectively the genes encoding their catalysts, have a higher propensity to be retained in the course of network evolution.

5. The predicted core metabolic graphs from parasites and *H. sapiens* were compared in a pharmacological context to identify candidate antiparasitic targets. Looking for metabolites of higher connectivity in parasites than in the host, there were much fewer than might have been expected based on the random networks. One such metabolite was threonine in *T. brucei*,

indicating that threonine antimetabolites could be worthwhile to test for specific antitrypanosomal activity.

6. Lists of core metabolic enzymes were compiled that are present in parasites but not in *H. sapiens*. There were extensive overlaps among kinetoplastid parasites, but not between the kinetoplastids and *P. falciparum*. Methionine metabolism in *T. brucei* was chosen for experimental investigation due to its pharmacological importance beyond core metabolism, i.e. the links to ergosterol synthesis and trypanothione synthesis.

7. Cobalamin-independent methionine synthase (Tb927.8.2610; EC 2.1.1.14) and homocysteine S-methyltransferase (Tb927.1.1270; EC 2.1.1.10) from *T. brucei* were validated as candidate drug targets in the sense that these enzymes are (i) absent from the human proteome; (ii) expressed in bloodstream-form trypanosomes; (iii) essential for growth of bloodstream-form trypanosomes at physiological levels (30 μM) of methionine.

Materials and methods

In silico reconstruction of metabolic networks

For all of the selected core metabolic pathways, educt-product pairs of reactions and the corresponding enzymes were retrieved manually from the Reference pathway maps of the KEGG database [35] and supplemented with data kindly provided by H. Ma and A.P. Zeng (German Research Center for Biotechnology, Braunschweig, Germany). Organism-specific enzyme lists were also obtained from KEGG, via LinkDB. The networks were reconstructed as directed graphs, but for all subsequent analyses treated as undirected. The basic network properties such as average connectivity or network diameter were determined with BioLayout [36]. The metabolites' connectivities and the connectivities' frequency distribution were determined with self-developed Perl scripts. Shrinkage of networks was performed with a Perl script that randomly removed educt-product pairs from metabolic maps until a given size was reached. These scripts are available upon request.

Reverse genetics with *Trypanosoma brucei*

Bloodstream-form *T. b. brucei* BS221 and procyclic-form *T. b. brucei* 427 were used for gene expression analysis. Total RNA was extracted with hot phenol, reverse transcribed with RetroScript (Ambion), and amplified by PCR using a forward primer (cgctattattagaacagttt) complementary to the 5' spliced leader sequence common to all trypanosomal mRNA [37], in combination with gene-specific reverse primers for methionine synthase Tb927.8.2610 (gccc<u>ctcgagggagttccgcgaaatattca</u>; MetE-rev, XhoI restriction site underlined), homocysteine methyltransferase Tb927.1.1270 (gcc<u>ctcgag</u>caacgacactcgcttcaaga), or *T. brucei* actin (ccacctgcataacattg).

Bloodstream-form *T. b. brucei* "New York single marker" (SM, [33]) were kindly provided by G.A.M. Cross (Rockefeller University). The gene Tb927.1.1270 was homozygously disrupted by sequential replacement, via homologous recombination, with constructs of the hygromycin and puromycin resistance markers, respectively, flanked by Tb927.1.1270 5' and 3' UTR sequences cloned into pBluescript. $4 * 10^7$ trypanosomes of the SM cell line were electroporated with 10 µg of linearized plasmid DNA, and positive transfectants were selected in HMI9 medium (BioConcept, Switzerland) supplemented with 1.0 µg/ml hygromycin, respectively 0.2 µg/ml puromycin. To silence the gene Tb927.8.2610, a stem-loop construct was cloned by ligating a 536 bp fragment of the gene twice, in opposite directions, into pSLcompl (kindly provided by I. Roditi and S. Hänni, University of Bern). The fragment had been amplified by PCR with the primers MetE-rev and MetE-fwd

(gcccggatccgacatcttgctcccttctgc: BamHI site underlined) and verified by sequencing. The RNAi construct was linearized with *Not*I, purified by ethanol precipitation, and transfected into the SM line by electroporation. Transfectants were selected with 1.0 µg/ml phleomycin in HMI9 medium. A double mutant cell line was generated by transfecting the methionine synthase knockout line (see above) with the above RNAi construct. Selection of positive transfectants was done using 1.0 µg/ml hygromycin, 0.2 µg/ml puromycin and 1.0 µg/ml phleomycin in HMI9 medium.

Methionine-free medium was prepared based on the recipe for Iscove Dulbecco's modified medium (Invitrogen) and supplemented with 10% fetal calf serum (Gibco-Bioconcpt) and 200 µM methionine (standard HMI9 medium), 30 µM methionine (physiological concentration), or no extra methionine. The RNAi cells were grown in the presence of 1.0 µg/ml tetracycline to induce expression of the stem-loop construct. Population doubling times were determined by linear regression against a log-scale growth curve.

Acknowledgements

We wish to thank H. Ma and A.P. Zeng for providing reaction files, G.A.M. Cross for the *T. b. brucei* NYSM line, and I. Roditi and S. Hänni for the plasmid pSLcompl. This work was supported by the Swiss National Science Foundation.

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Figure legends

Figure 1 - Core metabolic graphs: parasites vs. non-parasites

Graph properties of the predicted metabolic networks from parasites (red) and non-parasites (green). Cho, *Cryptosporidium hominis*; Ecu, *Encephalitozoon cuniculi*; Ehi, *Entamoeba histolytica*; Lma, *Leishmania major*; Pfa, *Plasmodium falciparum*; Tbr, *Trypanosoma brucei*; Tcr, *Trypanosoma cruzi*; Tpa, *Theileria parva*; Cal, *Candida albicans*; Cel, *Caenorhabditis elegans*; Ddi, *Dictyostelium discoideum*; Dme, *Drosophila melanogaster*; Eco, *Escherichia coli*; Hsa, *Homo sapiens*; Sce, *Saccharomyces cerevisiae*; Spo, *Schizosaccharomyces pombe*.

Figure 2 - Frequency distributions of connectivities

Double-logarithmic plot of the frequency distributions of connectivities by node (metabolite). The parasites are shown in red (n=8), the non-parasites in green (n=8), error bars indicate standard deviation. Grey line, reference network; dashed line, average of randomly shrunk networks (n=10).

Figure 3 - Comparative connectivities of core metabolites

For every degree (connectivity), the number of nodes (metabolites) in *T. brucei* (left) or *P. falciparum* (right) is plotted against the number of nodes in *H. sapiens*. The grey diagonal indicates metabolites of equal connectivity in host and parasite (AcCoA, acetyl-coenzyme A; DAG-AEP, diacylglyceryl-2-aminoethylphosphonate; Pyr, pyruvate).

Figure 4 - Parasite-specific enzymes

Enzymes (by E.C. number) present in parasites but not in their host *H. sapiens*. The absence of these enzymes in the predicted human proteome was verified by blastp searches.

Figure 5 - Methionine metabolism in Trypanosoma brucei

a) Different enzymes and methyl donors involved in methionine recycling from homocysteine in *H. sapiens* (green) and *T. brucei* (red). b) Methionine metabolism in African trypanosomes after [31]. Red, steps or pathways present only in *T. brucei*; green, steps or pathways present only in *H. sapiens*; grey, functions of methionine that are the same in host and parasite.

Tables

Table 1 - Species overview

Organism	Kingdom	Type	# Proteins	# Enzymes
Homo sapiens	Metazoa	F	25000	324
Escherichia coli	Bacteria	FP	4347	322
Candida albicans	Fungi	FP	6160	299
Saccharomyces cerevisiae	Fungi	F	6300	252
Schizosaccharomyces pombe	Fungi	F	4824	233
Dictyostelium discoideum	Protista	F	12500	228
Drosophila melanogaster	Metazoa	F	13600	217
Caenorhabditis elegans	Metazoa	F	19000	214
Trypanosoma cruzi	Protista	OP	22570	171
Leishmania major	Protista	OP	8010	163
Trypanosoma brucei	Protista	OP	9068	146
Plasmodium falciparum	Protista	OP	5300	139
Entamoeba histolytica	Protista	OP	9938	115
Theileria parva	Protista	OP	4079	97
Cryptosporidium hominis	Protista	OP	4000	69
Encephalitozoon cuniculi	Fungi	OP	1997	62

Species included in the study, their predicted numbers of protein-coding genes, and number of enzymes (as predicted by KEGG) used for reconstruction of core metabolic networks (OP, obligate endoparasite; F, free-living; FP, facultative parasite).

Table 2 - Basic graph properties

Network	Nodes	Edges	Avg. Connectivity	Max. Connectivity	Diameter	Isolated edges
Reference	876	1184	2.7	18	24	17
Non-parasites	483 ± 52	539 ± 79	2.2 ± 0.1	11.3 ± 1.5	25.6 ± 1.1	50 ± 9
Parasites	287 ± 67	278 ± 80	1.9 ± 0.1	8.3 ± 0.7	22.7 ± 7.1	51 ± 4
Random	333 ± 8.6	279 ± 7.0	1.7 ± 0.0	7.2 ± 1.5	15.4 ± 3.9	74 ± 6

Basic graph parameters (± standard deviation) of the reconstructed core metabolic networks, the randomly shrunk networks, and the reference network of all core metabolic reactions.

Table 3 - Genetic manipulation of methionine-recycling enzymes in T. brucei

	Methionine concentration in the medium:		
***T. brucei* line**	**200 µM**	**30 µM**	**0 µM**
Parental SM	10.0 h	24.4 h	no growth
SMΔmetE	15.7 h	no growth	no growth
SM–hcmRNAi	11.3 h	no growth	no growth
SM--ΔmetE–hcmRNAi	11.7 h	no growth	no growth

Population doubling times of bloodstream forms of the *T. b. brucei* SM parental line and transgenic descendants, in dependence of the methionine concentration in the medium. ΔmetE, knock-out of methionine synthase; hcmRNAi, knock-down of homocysteine methyltransferase; ΔmetE–hcmRNAi, double mutant.

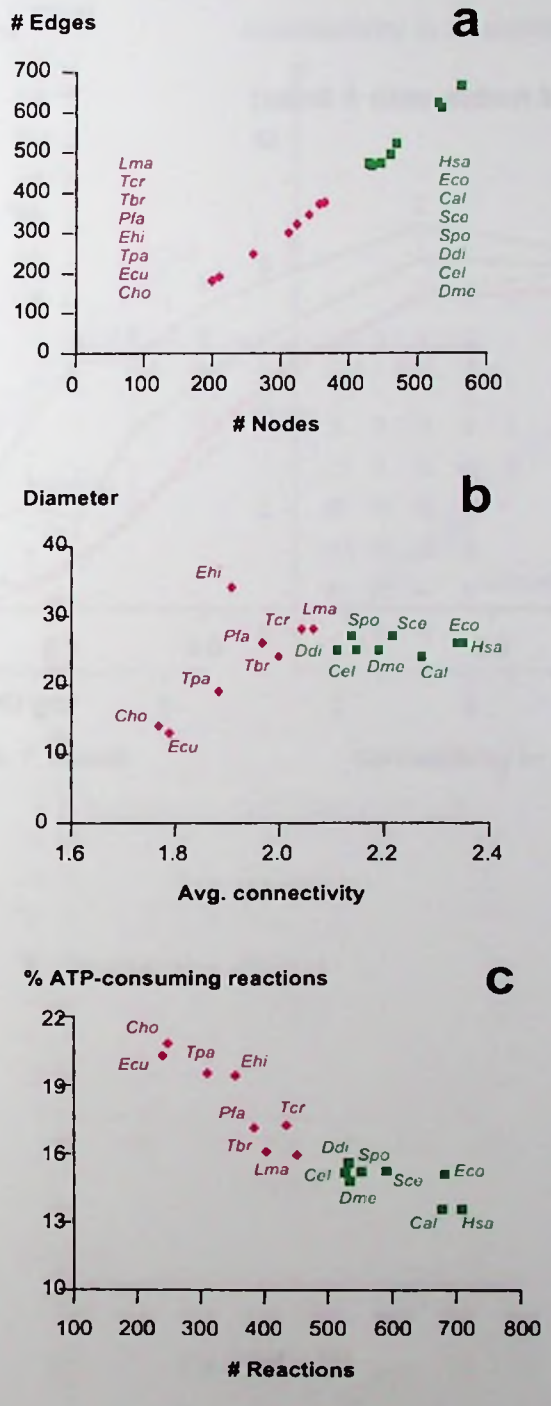
a
Edges
700
600
500
400
300
200
100
0
0
100
200
300
400
500
600
Nodes
Lma
Tcr
Tbr
Pfa
Ehi
Tpa
Ecu
Cho
Hsa
Eco
Cal
Sce
Spo
Ddi
Cel
Dme
b
Diameter
40
30
20
10
0
1.6
1.8
2.0
2.2
2.4
Avg. connectivity
Ehi
Tcr
Lma
Spo
Sce
Eco
Hsa
Pfa
Ddi
Tbr
Cel
Dme
Cal
Tpa
Cho
Ecu
c
% ATP-consuming reactions
22
19
16
13
10
100
200
300
400
500
600
700
800
Reactions
Cho
Ecu
Tpa
Ehi
Pfa
Tcr
Tbr
Ddi
Spo
Lma
Cel
Sce
Eco
Dme
Cal
Hsa

Figure 1

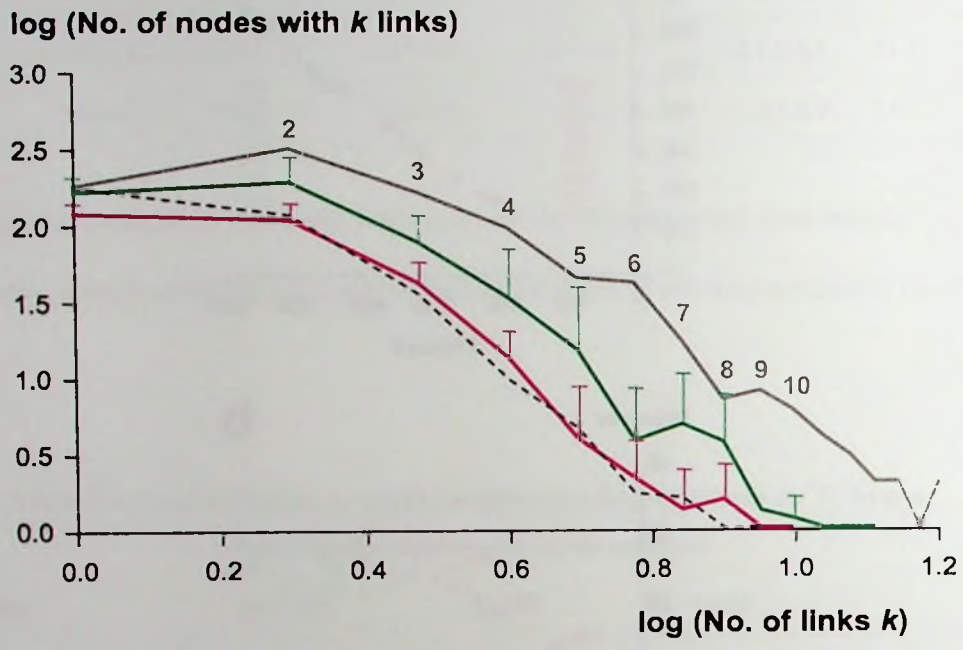
log (No. of nodes with k links)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
2
3
4
5
6
7
8
9
10
log (No. of links k)

Figure 2

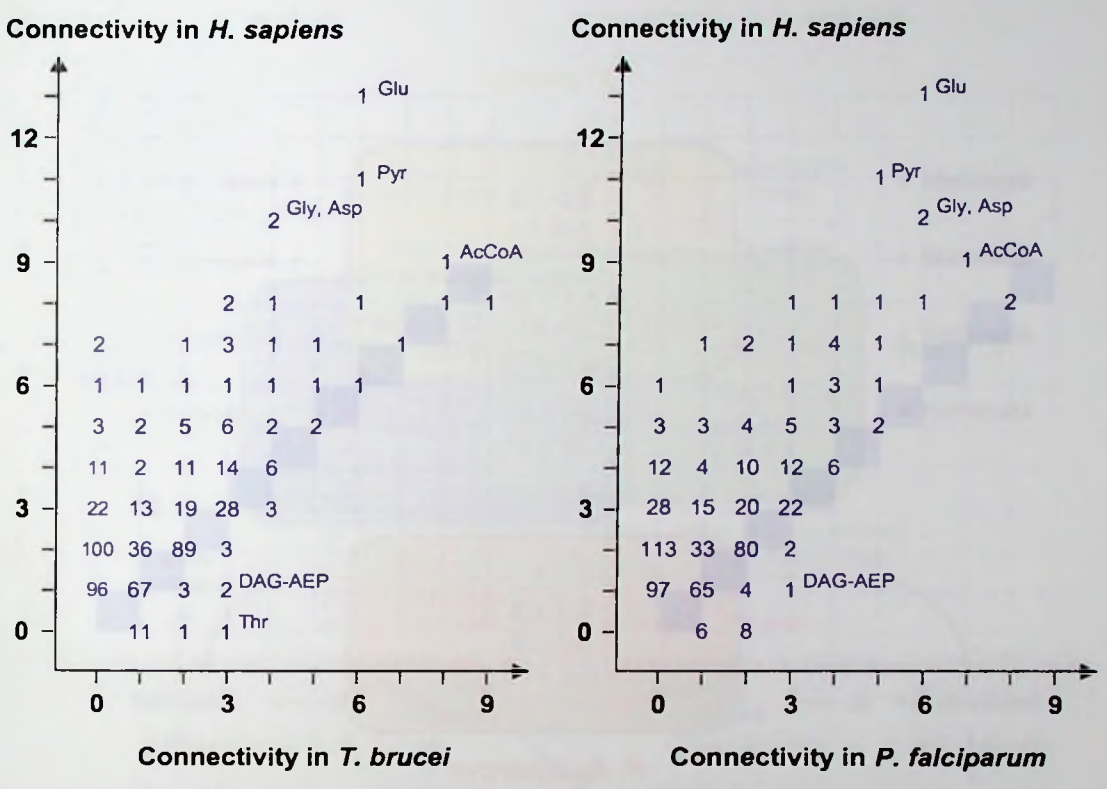
Connectivity in H. sapiens
Connectivity in H. sapiens
Glu
Pyr
Gly, Asp
AcCoA
DAG-AEP
Thr
0
3
6
9
12
Connectivity in T. brucei
Connectivity in P. falciparum

Figure 3

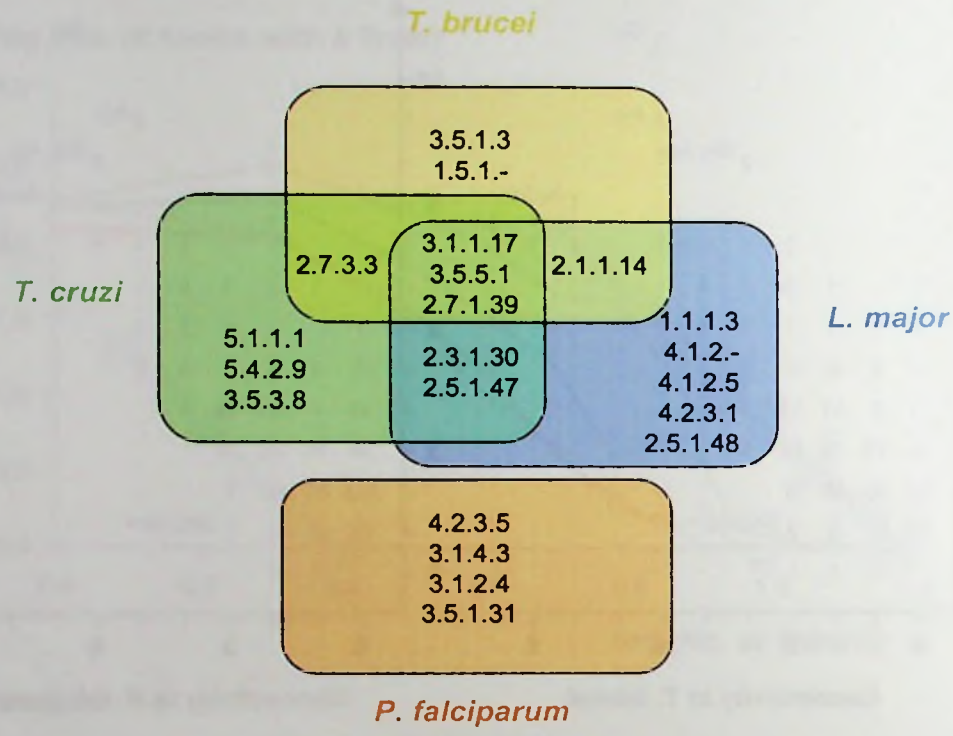
T. brucei
3.5.1.3
1.5.1.-
T. cruzi
2.7.3.3
3.1.1.17
3.5.5.1
2.7.1.39
2.1.1.14
L. major
5.1.1.1
5.4.2.9
3.5.3.8
2.3.1.30
2.5.1.47
1.1.1.3
4.1.2.-
4.1.2.5
4.2.3.1
2.5.1.48
4.2.3.5
3.1.4.3
3.1.2.4
3.5.1.31
P. falciparum

Figure 4

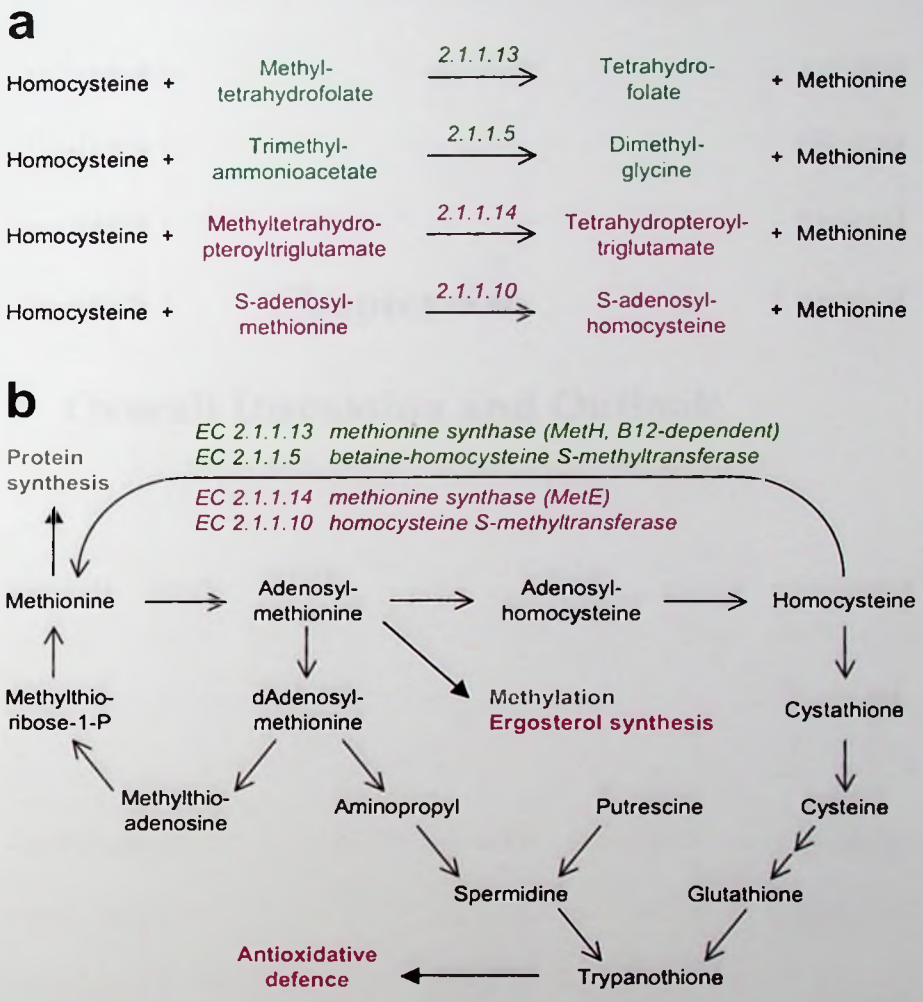
a
Homocysteine + Methyl-tetrahydrofolate 2.1.1.13 Tetrahydro-folate + Methionine
Homocysteine + Trimethyl-ammonioacetate 2.1.1.5 Dimethyl-glycine + Methionine
Homocysteine + Methyltetrahydro-pteroyltriglutamate 2.1.1.14 Tetrahydropteroyl-triglutamate + Methionine
Homocysteine + S-adenosyl-methionine 2.1.1.10 S-adenosyl-homocysteine + Methionine
b
EC 2.1.1.13 methionine synthase (MetH, B12-dependent)
EC 2.1.1.5 betaine-homocysteine S-methyltransferase
EC 2.1.1.14 methionine synthase (MetE)
EC 2.1.1.10 homocysteine S-methyltransferase
Protein synthesis
Methionine
Adenosyl-methionine
Adenosyl-homocysteine
Homocysteine
Methylthio-ribose-1-P
dAdenosyl-methionine
Methylation
Ergosterol synthesis
Cystathione
Methylthio-adenosine
Aminopropyl
Putrescine
Cysteine
Spermidine
Glutathione
Antioxidative defence
Trypanothione

Figure 5

Chapter 5.0

Overall Discussion and Outlook

Chapter 5.0

Overall Discussion and Outlook

Sleeping sickness is a human disease caused by two subspecies of *Trypanosoma brucei* (*Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*) and is transmitted by tsetse flies (Stich *et al*, 2002). Sleeping sickness occurs in rural areas of 36 countries in sub-Saharan Africa and it causes huge economic losses estimated at US$ 4.5 billion (WHO, 2002). Sleeping sickness disease management involves diagnosis, staging and chemotherapy. Diagnosis involves first the serological tests which are used for screening purposes, and parasitological tests which detect presence of the parasites and hence are used for confirmatory purposes (WHO, 2006). Sleeping sickness is a neglected disease with respect to chemotherapy as well as diagnostics. Diagnosis is either not sensitive enough or laborious while chemotherapy relies on a few drugs which are characterized by high toxicity, inefficiency and high costs (Brun and Balmer, 2006). In this work I address some of the problems associated with diagnosis and chemotherapy using both computational methods and laboratory experimentation. With the availability of complete genome sequences of different organisms, comparative genomics makes it possible to identify organism-specific genes or proteins that can be potential drug targets, vaccine targets, diagnostics antigens or virulence factors (Galperin and Koonin, 1999). One aspect of this thesis was to evaluate Tandem Repeat (TR) proteins as potential diagnostic antigens for sleeping sickness. TR proteins are often potent B-cell epitopes and their immunological significance has been reported for many infections (Allred *et al*, 1990; Burns *et al*, 1992; Gruber and Zingales, 1993; Bhatia *et al*, 1999; Burns *et al*, 1993; Coppel *et al*, 1984; Dame *et al*, 1984; Koenen *et al*, 1984; Müller *et al*, 1991). Traditionally, antigens are identified by serological screening of expression libraries (Knudsen and Vedeler, 2006; Jiang *et al*, 2008) or by immunobloting of crude cell lysate separated by two-dimensional gel electrophoresis. Computational identification of TR

proteins provides an additional tool for identifying potential antigens that can be used to develop diagnostic tools (Goto *et al*, 2008). Repetitive proteins were retrieved from the *T. brucei* predicted proteome v4 using the Dora database (Fankhauser *et al*, 2007). To get *T. brucei*-specific TR proteins, the retrieved proteins were blasted against proteomes of different organisms (Figure 1) with the aim of getting rid of antigens that cross-react and could result in false positives. Of the 19 proteins that were obtained by the above procedure, three (Tb09.211.1800, Tb10.406.0650, and Tb10.70.6570) were selected for expression as recombinant proteins and evaluation using ELISA. Results show that the selected antigens are not specific since they cross-react with sera of healthy individuals from sleeping sickness endemic areas. Previous studies also show that MARP1 (Tb10.406.0650) and I/6 were recognized by self-reactive antibodies of uninfected mice (Müller *et al*, 1992). These results emphasize the fact that computational prediction alone is not sufficient to identify potential antigens but is a tool that provides an initial filter to screen for potential antigens that are further validated by wet-laboratory experimentation. In addition, there is need to evaluate more TR proteins. However, since the number of sera used in our study is too small to make conclusive remarks about the antigens, we have submitted them to FIND (Foundation for Innovative New Diagnostics) Geneva, for further evaluation.

Chemotherapy is the principle control method for sleeping sickness since the parasites evade the host immune system through antigenic variation (Turner, 1999). However, the current therapy is not satisfactory since it relies on a few drugs which are expensive, toxic and inefficient (Brun and Balmer, 2006). Drug resistance for sleeping sickness chemotherapy is mainly reported for melarsoprol although cross-resistance to pentamidine also occurs (Rollo and Williamson, 1959). Understanding the mechanisms of drug resistance is essential for the control of diseases (Ouellette, 2002). Forward and reverse genetics offer useful tools that can

be used to study drug resistance. In this work, both methods were used as described in chapter 3A reverse genetics), 3B and 3C (forward genetics). Resistance to melarsoprol in the field is partly associated with loss of activity in an aminopurine (P2) transporter (Matovu *et al*, 2003), one of the uptake routes of melaminophenyl arsenicals and diamidines into the parasite (Carter and Fairlamb, 1993; Mäser *et al*, 1999; de Koning, 2001a). Laboratory studies have shown that loss of TbAT1 results in low level resistance to melarsoprol suggesting that high level resistance is associated with at least one other transporter for example HAPT1. However, genetic manipulation of HAPT1 to investigate its role in melarsoprol resistance is not possible because the gene encoding this transporter is not known. Another mechanism associated with melarsoprol resistance in *T. brucei* but only known in the laboratory is overexpression of the ABC-type efflux pump, TbMRPA (Shahi *et al*, 2002). One of the aims here was to investigate what happens when the two mechanisms, loss of P2 transporter and overexpression of TbMRPA, coincide in the same cell. Results (Chapter 3a) show that the contributions of the two mechanisms to drug resistance are simply additive. However, loss in sensitivity caused by the two mechanisms could be sufficient to cause treatment failure in the field. Results from this study also show that there is a possibility of cross-resistance between melarsoprol and diamidines due to overexpression of TbMRPA because overexpression of TbMRPA led to a significant loss of sensitivity of the parasites to berenil as compared to the wildtype. To further investigate cross-resistance between melarsoprol and diamidines, we used isolates that were independently selected for resistance against pentamidine and melarsoprol. Results obtained confirm the roles of TbAT1, HAPT1 and LAPT1, in high level resistance of trypanosomes to melarsoprol and pentamidine. This is in agreement with the fact that parasites use multiple means to achieve drug resistance (Ouellette, 2002). To identify the actual genes involved in the resistance profiles exhibited by these isolates we suggest that

other techniques like Microarray analysis or genome sequencing be used to compare sensitive isolates and their resistant derivatives.

One way of managing drug resistance is the identification of new drug targets. With the availability of fully sequenced genomes, potential drug targets can be identified by comparative genomics (Galperin and Koonin, 1999). The advantage with this approach is that the drug's target is known in advance thus providing a rational base for drug combinations (Mäser, December 2008). Parasites have lost many metabolic reactions or pathways in the course of evolution. However, they have also retained certain reactions which are missing in the host and therefore can be targeted by drugs. We compared core metabolism of parasites and that of non-parasites with the aim of determining how networks shrink and identifying potential drug targets. Results (Chapter 4.0) show that loss of pathways by parasites is not a random event. This is in agreement with the fact that parasites co-evolve with their hosts and hence their metabolism is defined by their ecological niche (Ginger, 2006). However, it is also important to note that although parasites, like their hosts, have retained certain pathways, certain aspects of those pathways are different. For example salvage of methionine from homocysteine is catalysed by methionine synthase (EC2.1.1.13) and betaine-homocysteine methyltransferase (EC2.1.1.5) in the human host and catalysed by methionine synthase (EC2.1.1.14) and homocysteine methyltransferase (EC2.1.1.10) in *T. brucei*. Methionine synthase (EC2.1.1.13) is Vitamin B12-dependent while *T. brucei* methionine synthase (EC2.1.1.14) is Vitamin B12-independent. We investigated the importance of such parasite-specific enzymes by knocking out homocysteine methyltransferase and found that homocysteine methyltransferase is essential for growth of *T. brucei* in medium supplemented with 30 μM methionine. Knockdown of methionine synthase did not yield conclusive results since the RNAi seems to have been leaky. We therefore recommend to use a knockout of

methionine synthase instead of the knockdown and also to investigate the infectivity of the mutant parasites *in vivo*. However, since *T. brucei* are auxotrophic for methionine (Chapter four), methionine metabolism is a potential pathway for drug targeting.

Overall, with availability of fully sequenced genomes, comparative genomics is a useful and fast approach for identification of potential antigens, vaccine targets or drug targets. In addition, forward and reverse genetics offer useful tools that can be used to generate information on the behaviour of parasites under different conditions and hence provide information that can be used in disease control. Finally, *in silico* methods should always be supplemented with laboratory experimentation to obtain conclusive remarks about a predicted target.

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Appendix

Appendix

Methionine-free medium

Compound	MW	USED*	mg/L	Molarity mM
Nom Essential amino acids				
1. L-Alanine	89		25	0.281
2. L-Asparagine	150		25	0.167
3. L-Aspartic acid	133		30	0.226
4. L-Glutamic acid	147		75	0.510
5. Glycine	75		30	0.400
6. L-Proline	115		40	0.348
7. L-Serine	105		42	0.400
Essential amino acids				
8. L-Arginine hydrochloride	211	L-Arginine (174.2)	69.3	0.398
9. L-Histidine Hydrochloride-H2O	210	Histidine (155.16)	31	0.200
10. L-Isoleucine	131		105	0.802
11. L-Leucine	131		105	0.802
12. L-Lysine hydrochloride	183	Lysine (146.2)	116.7	0.798
13. L-Phenylalanine	165		66	0.400
14. L-Threonine	119		95	0.798
15. L-Tryptophan	204		16	0.0784
16. L-Tyrosine disodium salt	225	L-Tyrosine (181.19)	83.7	0.462
17. L-Valine	117		94	0.803
18. L-Glutamine	146		584	4.00
19. L-cystine 2HCl			91.4	0.381
Vitamins				
1. Biotin	244		0.013	0.0000533
2. Choline chloride	140		4	0.0286
3. D-Calcium pantothenate	477		4	0.00839
4. Folic acid	441		4	0.00907
5. I-inositol (myo-inositol)	180		7.2	0.0400
6. Niacinamide (Nicotinamide or Vit B3 or Niacin)	122	Nicotinamide	4	0.0328
7. Pyridoxal hydrochloride	204		4	0.0196
8. Riboflavin	376		0.4	0.00106
9. Thiamine hydrochloride	337		4	0.0119
10. Vitamin B12	1355	10 µl of 1.52mg in 1.169ml water	0.013	0.00001
Inorganic salts				
1. Calcium chloride (anhydrous)	111	CaCl2.2H20 (147.02)	218.6	1.49
2. Magnesium sulfate (anhydrous)	120	MgSO4 heptahydrate (246.48)	200.6	0.814
3. Sodium chloride	58		4500	77.59
4. Sodium Phosphate monobasic (NaH2PO4-H2O)	138		125	0.906
5. Sodium selenite (Na2SeO3-5H2O	263	Na2SeO3 (172.94)- 1.5 µl of 9.4mg in 1.237ml water.	0.0114	0.000066
Potassium chloride	74.55		165	2.2
Potassium nitrate	101.1	10 µl of solution (9.4 mg in 1.237ml) water.	0.076	0.000752
Other components				
1. D-Glucose (Dextrose)	180		4500	25.0
2. HEPES	238		5958	25.03
3. Phenol Red	376.4		15	0.0399
4. Sodium pyruvate	110		110	1.000
5.NaHCO3	84.01		302.4	3.6
6. Thymidine	242.2		39	0.16

*Indicated if what was specified was not available.

2. Dissoloved in 800ml MiliQ water, stirr for 1 hour
3. Adjust pH to 7.30
4. Filter to sterilise and store at 4^0C

To the above add the following
1. 14.2ul of beta Mercaptoethanol. Final concentration is 0.2mM
2. 10ml of 100X Bathocuprain
3. 10ml of 100X Hypoxanthine
4. 182mg of L-Cysteine.
5. 100ml Fetal calf serum.
6. 10ml penicillin/streptomycin stock (this can be added here or in the stock).
7. Top up to 1 litre with MilliQ water.

Filter to sterilize.

Declaration of Originality

Declaration of Originality

Last name, first name: **Nerima Barbara**

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I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date Signature

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Curriculum Vitae

Curriculum Vitae

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