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NOVEL TREATMENT OF AFRICAN TRYPANOSOMIASIS

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Stockholm 2013

Published and printed by Larserics Digital Print AB, Sundbyberg.

ISBN 978-91-7549-030-4

ABSTRACT

Human African Trypanosomiasis (HAT) or Sleeping Sickness is fatal if untreated. Current drugs used for the treatment of HAT have difficult treatment regimens and unacceptable toxicity related issues. The effective drugs are few and with no alternatives available, there is an urgent need for the development of new medicines, which are safe, affordable and have no toxic effects. Here we describe different series of lead compounds that can be used for the development of drugs to treat HAT.

In vivo imaging provides a fast non-invasive method to evaluate parasite distribution and therapeutic efficacy of drugs in real time. We generated monomorphic and pleomorphic recombinant *Trypanosoma brucei* parasites expressing the *Renilla* luciferase. Interestingly, a preferential testis tropism was observed with both the monomorphic and pleomorphic recombinants. Our data indicate that preferential testis tropism must be considered during drug development, since parasites might be protected from many drugs by the blood-testis barrier **(Paper I)**.

In contrast to most mammalian cells, trypanosomes cannot synthesize purines *de novo*. Instead they depend on the host to salvage purines from the body fluids. The inability of trypanosomes to engage in de novo purine synthesis has been exploited as a therapeutic target by using nucleoside analogues. We showed that adenosine analogue, cordycepin in combination with deoxycoformycin cures murine late stage models of African trypanosomiasis **(Paper II)**.

Since deoxycoformycin was shown to be a teratogen, we aimed for developing deoxycoformycin independent deaminase resistant cordycepin analogues that could be used as standalone drugs in treatment for African trypanosomiasis. We synthesized and characterized several deaminase resistant cordycepin analogues. 2-Fluorocordycepin (2-fCy) showed selective trypanotoxicity and resistance against adenosine deaminase. 2-fCy showed good in vitro preclinical profile and is a promising new lead for development of treatment against African trypanosomiasis **(Paper III)**.

In order to identify new drugs for the treatment of HAT we performed a focused screen of 5,500 compounds for *Trypanosoma brucei* subsp. A number of 2-aminopyrazines/2-aminopyridines were identified as promising leads. Specifically, CBK201352 a 2-aminopyrazine compound was trypanotoxic for *T. brucei*. In vitro preclinical assays predicted that CBK201352 has promising pharmacokinetic parameters. Mice treated with CBK201352 showed complete clearance of parasites for more than 90 days. Thus, we show that CBK201352 and related analogs are promising leads for the development of novel treatments for HAT **(Paper IV)**.

The intracellular reducing environment of trypanosomes is maintained by a unique thiol system. In trypanosomes the trypanothione and trypanothione reductase (TryR) replace glutathione and glutathione reductase found in most other organisms. TryR is an essential enzyme for the parasite and is absent in mammalian cells making it an attractive drug target. We showed that ebsulfur (EbS) a small sulfur-containing molecule is a NADPH, concentration and time-dependent irreversible inhibitor of the *T. brucei* TryR. We demonstrated that EbS or analogues disrupt the trypanothione system with a novel mechanism and are promising lead compounds for the development of drugs to treat HAT **(Paper V)**.

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I. Claes F, **Vodnala SK**, Van Reet N, Boucher N, Lunden Miguel H, Baltz T,Goddeeris BM, Büscher P, Rottenberg ME. (2009)
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PLoS Negl Trop Dis 3(7): e486. doi:10.1371/journal.pntd.0000486.

II. **Vodnala SK**, Ferella M, Lundén-Miguel H, Betha E, Van Reet N, Amin DN, Oberg B, Andersson B, Kristensson K, Wigzell H, Rottenberg ME. (2009).
"Preclinical Assessment of the Treatment of Second-Stage African Trypanosomiasis with Cordycepin and Deoxycoformycin."
PLoS Negl Trop Dis 3(8): e495. doi:10.1371/journal.pntd.0000495.

III. **Vodnala SK**, Yeheskieli E, Lundbäck T, Sjöberg B, Svensson R, Hammarström L G J, Rottenberg ME.
"Characterization of adenosine deaminase-resistant cordycepin analogues as leads for treatment of African trypanosomiasis."

*Manuscript

IV. **Vodnala SK**, Lundbäck T, Sjöberg B, Svensson R, Rottenberg ME, &Hammarström L G J (2012).
"In vitro and In vivo Activity of 2-Aminopyrazines/2-Aminopyridines in Experimental Models of Human African Trypanosomiasis."
Antimicrobial agents and chemotherapy. doi:10.1128/AAC.01870-12.

V. Lu J*, **Vodnala SK*,** Gustavsson AL, Gustafsson TN, Sjöberg B, Johansson HA, Sangit K, Tjernberg A, Lars Engman, Rottenberg M E[#], and Holmgren A[#].
"Ebsulfur: a novel *Trypanosoma brucei* cytocidal inhibitor targeting the trypanothione metabolism."
*Manuscript

Publications outside the scope of the thesis

1. Amin D N, **Vodnala SK**, Masocha W, Sun B, Kristensson K, & Rottenberg ME. (2012).
   "Distinct Toll-like receptor signals regulate cerebral parasite load and interferon α/β and tumor necrosis factor α-dependent T-cell infiltration in the brains of Trypanosoma brucei-infected mice."
   The Journal of infectious diseases, 205(2), 320–32. doi:10.1093/infdis/jir734

2. Cloete TT, Johansson CC, N'Da DD, **Vodnala SK**, Rottenberg ME, Breytenbach J C, & Ashton M. (2011).
   "Mono-, di- and trisubstituted derivatives of eflornithine: synthesis for in vivo delivery of DL-alpha-difluoromethylornithine in plasma."
   Arzneimittel-Forschung, 61(5), 317–25. doi:10.1055/s-0031-1296205

3. Rouf SF, Ahmad I, Anwar N, **Vodnala SK**, Kader A, Römling U, & Rhen M. (2011).
   "Opposing contributions of polynucleotide phosphorylase and the membrane protein NlpI to biofilm formation by Salmonella enterica serovar Typhimurium."
   Journal of bacteriology, 193(2), 580–2. doi:10.1128/JB.00905-10

LIST OF ABBREVIATIONS

AK	Adenosine Kinase
APRT	Adenine phosphoribosyltransferase
BBB	Blood brain barrier
CATT	Card Agglutination Test for Trypanosomiasis
CNS	Central nervous system
CSF	Cerebro spinal fluid
ES	Expression site
GSH	Glutathione
HAPT	High affinity pentamidine transporter
HAT	Human African trypanosomiasis
HPRT	Hypoxanthine phosphoribosyltransferase
HTS	High throughput screening
IVIS	*In vivo* imaging system
LAPT	Low affinity pentamidine transporter
LDL	Low-density lipoprotein
MTA	Methylthioadenosine
MTD	Maximum tolerated dose
MTRP	Methylthioribose-1-phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NECT	Nifurtimox-Eflornithine Combination chemotherapy
ODC	Ornithine decarboxylase
PCD	Programmed cell death
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TryR	Trypanothione reductase
TryS	Trypanothione synthetase
$T(SH)_2$	Trypanothione
VSG	Variant surface glycoprotein

TABLE OF CONTENTS

1. INTRODUCTION

African trypanosomiasis or sleeping sickness is a vector borne disease caused by subspecies of the parasitic protozoa *Trypanosoma brucei.* The disease is transmitted to humans, cattle and wild animals by the bite of tsetse fly (*Glossina* genus) and is endemic or 'foci' to sub-Saharan Africa, within the geographic distribution of the tsetse fly. Unlike other trypanosomatids, *Trypanosoma brucei* is an extracellular parasite, which lives and multiplies extracellularly in blood and tissue fluids. Human African trypanosomiasis (HAT) is caused by infection with two subspecies of *Trypanosoma brucei* (i.e. *T.b. gambiense* and *T.b. rhodesiense).*

There are two stages of infection in HAT. In early stage the parasites multiply in the haemolymphatic system. In the late stage parasites invade central nervous system (CNS) triggering neurological disorders leading to disturbances in sleep/wake pattern[1,2], which established the common name "sleeping sickness".

The vast majority (95%) of the reported cases of sleeping sickness in humans are due to gambian form (*T.b. gambiense*), causing chronic infection that can last for years, prevalent in Western and Central Africa[3]. The remaining 5% of cases are due to *T.b. rhodesiense,* with a more rapid outcome, which is dominant in Eastern and Southern Africa, where it also infects wild and domestic animals[3].

The other subspecies of trypanosomes such as *T. congolense, T. vivax, T. evansi* only cause animal African trypanosomiasis or "Nagana" in Zulu language[4]. The pathogenicity of the infection is relatively mild in wild animals, but is fatal in domestic animals. With the progression of the disease, domesticated cattle become weak and unfit for work, hence the name "N'gana" which is a Zulu word meaning "powerless/useless"[4].

Human and animal trypanosomiasis together are a major cause for the underdevelopment of many rural areas in sub-Saharan Africa[5]. Approximately 70 million people are estimated to be at HAT risk according to the recent geospatial analysis[6]. There is no vaccine available for HAT, as the parasites can change their surface coat protein, which allows them to evade the immune system[7,8]. At present, there are only 4 licensed drugs and one combinational therapy used for the treatment of HAT. Most of these drugs have limitations with adverse drug related toxicity issues and difficult treatment regimens. With the emergence of resistance to current drugs and no

backup drugs available, there is an urgent need to develop new drugs for the treatment of HAT.

1.1 Life cycle

Tsetse flies of *Glossina* genus are the primary biological vectors, which transmit trypanosomes from one mammalian host to another. Transmission of the disease can occur by both male and female tsetse flies that have fed on infected blood. During the course of the developmental cycle, trypanosomes alternate between proliferative and non-proliferative forms. Proliferative or slender forms divide by binary fission and non-proliferative or stumpy forms are incapable of division[9].

With the bite of an infected tsetse fly, trypanosomes enter through the wound reaching the draining lymphatics and later multiply in the bloodstream[9]. In the bloodstream trypanosomes become polymorphic, comprising of dividing long slender forms and non-dividing intermediate and short stumpy forms (Fig.1). Short stumpy forms have characteristics of cell cycle arrest, increased mitochondrial activity and resistance to antibody responses[10–12]. Short stumpy forms will pre adapt for transition into procyclic forms .The non dividing short stumpy forms are in turn taken in a blood meal of tsetse fly to complete the life cycle[9].

Upon feeding on infected blood, stumpy forms enter into the lumen of the midgut of the tsetse fly where it transforms into a procyclic stage[9]. Slender forms taken with the blood meal cannot survive inside the insect. The procyclic forms adapt to the insect environment by switching the energy metabolism from glucose to proline as the principal energy source[9]. Procyclic forms undergo several differentiation steps in the gut and migrate to the salivary glands to transform into epimastigotes. In the salivary glands the epimastigotes undergo differentiation and mature into infective metacyclic forms (Fig.1)[13].

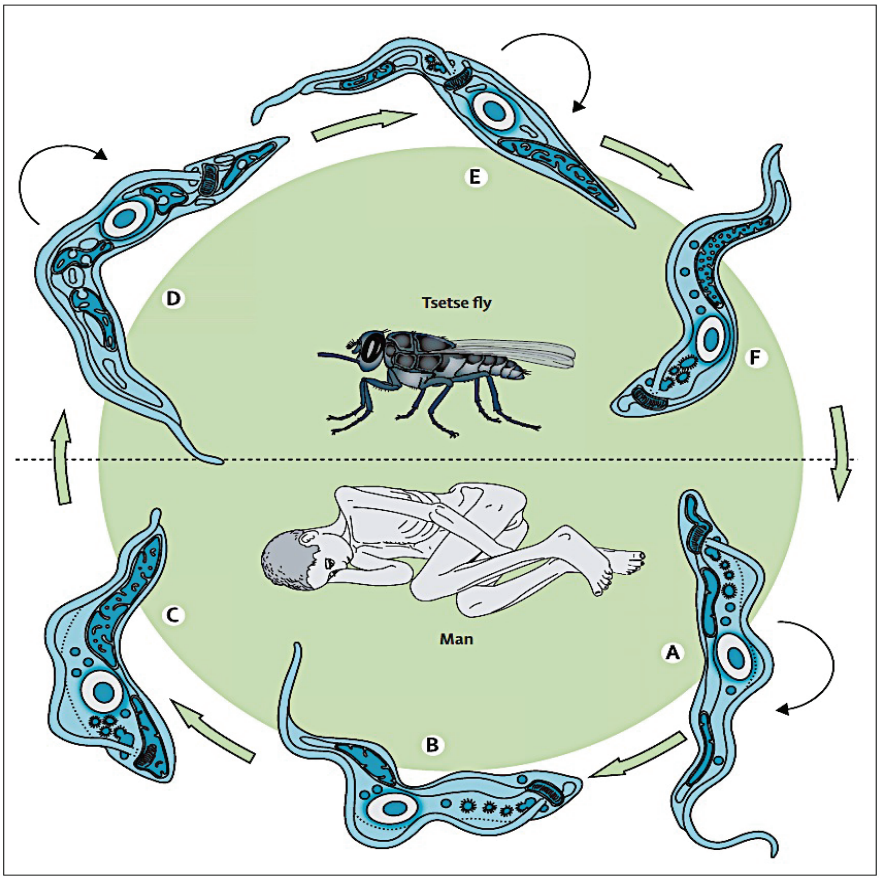


Fig 1. **Life cycle of African trypanosomes.** The lower half of the picture represents different phenotypes in man **A)** After the bite of an infected tsetse fly long slender forms multiply in blood and lymph **B&C)** Intermediate and short stumpy forms are the non-dividing forms. The upper half of the picture represents the differentiation of the trypanosomes in the tsetse fly **D)** The stumpy forms differentiate into procyclic dividing forms in the gut of the insect after infected blood meal **E)** After dividing in the gut some migrate to the salivary glands (procyclic forms) **F)** Maturation of epimastigote forms in the salivary glands to infective metacyclic trypomastigote forms. Arrow mark indicates the dividing forms. Picture adapted from Reto Brun *et al*[13].

1.2 Antigenic variation and Immune evasion

The antigenic variation in parasites is a prerequisite to sustain a long-term infection. The escape of humoral immune responses by constantly shedding major surface antigen, the variant surface glycoprotein (VSG) is a common phenomenon in the different animal and human forms of trypanosomiasis[14]. VSG is transcribed from a telomeric expression

site (ES) located at end of the chromosome. There are 20 ES situated on different telomeres, but allelic exclusion in trypanosomes will safeguard that only one VSG gene is transcribed at any given time from the ES[15]. Trypanosomes posses a large repertoire (>1000) of potential VSG genes in non-telomeric chromosome regions, and a certain transcribed VSG gene at the active ES can be replaced by a different VSG gene by gene conversion or reciprocal recombination into the telomeric ES[8,15]. During the course of infection, parasite numbers fluctuate as a result of host B and T cell responses, but the infection persists due to the variant VSG forms[14]. A definite number in changing VSG for a clone infection is unknown, but is approximately between 10^2-10^3 [9,16]. The process continues with peaks and drops of parasitemia leading to chronic infection, which is a typical characteristic of antigenic variation seen in African trypanosomiasis. Antigenic variation of VSG is sufficient to evade the immune responses in non-human primates.

In humans and most primates the defense against animal African trypanosomes is mediated by trypanosome lytic factor (TLF)[17–19]. Therefore animal forms of trypanosomes are not infective to humans. However human infectious forms, *T.b. gambiense* and *T.b. rhodesiense* are resistant to trypanolytic serum complexes by reducing the uptake of TLF[20,21] and neutralizing the effect of TLF by serum resistance associated (SRA) gene[22–24].

In the course of evolution, trypanosomes have not only evolved in mechanisms to dodge TLF and adapted to the changing hostile environment by antigenic variation, they also manipulate the immune responses of the host by secreting several parasite-derived factors[14].

1.3 Clinical symptoms

The infection with *T.brucei* is characterized by two stages, the early haemolymphatic stage (first stage) and the late meningoencephalitic stage (second stage). The severity, progression and symptoms of the disease are also dependent on the parasite species and the genetics of parasite and the host[25,26].

The course of infection with *T.b. rhodesiense* is more rapid than that of *T.b. gambiense* with death occurring in three weeks to two months of infection[27]. The mean average of the disease with *T.b. gambiense* is approximately three years[28]. It is reported that few cases of the Gambian form are characterized as asymptomatic when diagnosed

by the classical parasitological methods[29]. Such asymptomatic patients are serologically positive with no detectable parasites in the blood for several years.

1.3.1 Early haemolymphatic stage (first stage)

Following the bite of the tsetse fly, the infective metacyclic forms are deposited under the dermal connective tissue developing a local skin reaction. The chancre develops due to trypanosome proliferation and the inflammatory responses[30]. Superficial trypanosomal rashes or trypanosomal chancre on the skin can be seen as a result of tsetse fly bite, which is more frequently seen in travellers than in patients from endemic regions[25]. The reaction at the site of tsetse fly bite is rarely seen with *T.b. gambiense*, but detected in a fraction of the patients infected with *T.b. rhodesiense*[13]. The main symptoms of first stage are intermittent fever, headache, lymphadenopathy, pruritus and to a lesser extent hepato-splenomegaly.

1.3.2 Late meningoencephalitic stage (second stage)

The second stage is characterized by the invasion of parasites into the central nervous system (CNS). Parasites can actively cross the blood brain barrier and penetrate into the brain[31]. In the second stage of infection neurological disturbances like sleep, sensory, motor and neuro-psychiatric disorders are the more dominant symptoms[32]. Dysregulation of the sleep/wake cycle and fragmentation of the sleep pattern is the cause for sleeping disorders[33]. A wide spectrum of neurological disorders are involved with sleeping sickness and not all features are observed in a single patient. In brief neurological disorders include limb and tongue tremor, muscle fasciculation's, slurred speech, cerebral ataxia and abnormal movements[1]. Neurological disorders are not seen in the early stage and increase with the duration of the infection.

Cardiac related symptoms, as defined by ECG (electrocardiogram) alterations can be registered early in the evolution of the disease. The electrical cycle of the heart is measured by counting the time interval between starting Q and ending T wave. Prolonged QTc interval comprises a risk for fatal arrhythmia. Recent findings state that prolonged QTc intervals are more pronounced in early and late stage HAT patients than in healthy controls. However there are no cases reported with congestive heart failure in HAT[34].

1.4 Diagnosis

HAT diagnosis is primarily made by serological analysis and microscopic examination of lymph node aspirates, blood and/or cerebro spinal fluid (CSF). Diagnosis in HAT follows a three-step pathway: screening, diagnosis confirmation and staging.

Screening: In a mass population screening campaign, a "Card Agglutination Test for Trypanosomiasis" (CATT) is used in most areas of endemic infection. A drop of blood is used to check if it is HAT positive or negative. CATT is practical to use in rural areas and is cost effective. Ten samples can be tested at the same time and the result can be visualized by naked eye[35]. Unfortunately, CATT test does not react with *T.b. rhodesiense,* hence field screening in East Africa still relies on clinical symptoms. The other alternatives such as immunofluorescence and ELISA methods are used to screen patients under the expertise of trained professionals at clinical treatment centers[36].

Diagnosis Confirmation: CATT or serologically positive patients will be later examined microscopically for the presence of trypanosomes in blood, lymph nodes aspirates and CSF[35]. Apart from microscopical examinations, other extended diagnostic procedures like amplifying the *T.brucei* DNA using PCR, to increase the sensitivity are also used[37]. Microhemotocrit centrifugation technique (mHCT) and mini-anion-exchange centrifugation technique (mAECT) are a used to separate less negatively charged trypanosomes from blood cells over a chromatographic column and collected on a glass slide by spinning the column at low speed centrifugation[38,39]. These "concentration" techniques are time consuming but will increase the sensitivity in diagnosing trypanosomiasis when there are low levels of parasitemia.

Stage determination: According to WHO recommendations, second stage HAT is defined by the presence of a) raised white blood count (>5cells/μl)[40] b) presence of trypanosomes c) increased protein content (>370mg/liter)[40] in the CSF. The WBC threshold limit 5-cells/μl is controversial and threshold limit varies between countries. In general, patients with 6-20 cells/μl in CSF are referred as "early second stage" or "intermediate stage" of the disease[35,41].

Diagnosis play a important role in further spread or control of infection, since misdiagnosed individuals serve as potential reservoirs of infection for longer periods[29]. Despite few improvements achieved in the field of diagnosis, sensitive and simple serological diagnosis for both subspecies, especially *T.b. rhodesiense* are still lacking.

1.5 Treatment

The selection of treatment is decided upon determining the stage of the disease. There are very few drugs available for the treatment of HAT, some of them were already discovered in the early 20th century, presenting toxic effects and difficulties in administration. The first line drugs used for the treatment are suramin, pentamidine for early stages and melarsoprol, eflornithine for late stages. The late stage HAT is a consequence of the invasion of parasites in the CNS, hence any drug that is used for treating late stages must reach the parasite crossing the blood brain barrier (BBB). Many drugs that have potent trypanocidal activity in early stage models fail to cure late stage models due to difficulties in crossing BBB. Melarsoprol and eflornithine can cross the BBB and are used for the late stage treatment. Recently, a combination of nifurtimox and eflornithine (NECT) was introduced as an alternative for the late stage treatment. NECT was proposed as the best therapeutic option for the treatment of late stage gambiense HAT due to reduced dosage and treatment time[42,43]. The disadvantage of eflornithine is that it is ineffective for *T. b. rhodesiense,* thus melarsoprol remains the only option for the late stage treatment against *rhodesiense* infections. A detailed description of these drugs and summary is described in sections below.

1.5.1 Drugs used for early stages

Suramin

Suramin, a polysulphonated symmetrical naphthalene derivative was first used against HAT in 1922[44]. Use of synthetic dyes for the treatment of trypanosomiasis was first exploited by Paul Ehrlich[44]. Optimization of a series of synthetic dyes (e.g. Trypan blue, Fig.2) lead to the development of suramin (Fig.2)[44]. Suramin is used in clinics and is efficient to treat early stage HAT caused by both subspecies[13]. Usage of suramin is avoided against gambiense infections in Western Africa due to the presence of *Onchocerca* spp, since it increases the risk for severe allergic reactions in patients[13]. Hence treatment with suramin is restricted only to rhodesian forms.

Suramin is a negatively charged molecule that binds to many serum proteins including low-density lipoprotein (LDL). Trypanosomes endocytose proteins from the pocket at the base of the flagellum[45]. Probably, the complex of suramin and LDL is taken up by the parasite[46–48]. The mechanism of action of suramin is not completely understood, but is assumed that suramin targets glycolytic enzymes[49], because procyclic

forms are hundred fold less sensitive to this drug compared to blood stream forms. Glycolysis is not essential for procyclic but essential for blood stream forms[47,50].

Suramin gets rapidly degraded when exposed to air and has to be administered immediately after dissolving in distilled water[13]. Suramin is given intravenously (iv), and the regimen dose is complex, which last up to 30 days. Treatment with suramin results in frequent adverse drug reactions, usually mild and reversible, including nephrotoxicity, peripheral neuropathy and thrombocytopenia[51]. Rare acute and late hypersensitivity can occur, for which low test dose is given before treatment initiation[13].

Summary of Suramin

Treatment: Only for early stages.

Limitations: Cannot cross BBB.

Mode of action: Not known, multiple targets[52] (can interfere in polyamine pathway, trypanothione pathway, glycolytic enzymes[49]).

Resistance in field: Not observed.

Uptake: Endocytosis.

Dosing: Intravenous infusion, when test dose 4-5mg/kg is tolerated well, then 20mg/kg is given on 1,3,7,14,and 21 days (adults) or on day 3, 10,17, 24 and 31[13].

Side effects: nephrotoxicity, peripheral neuropathy, thrombocytopenia and adverse drug related acute and late hypersensitivities.

Structure:

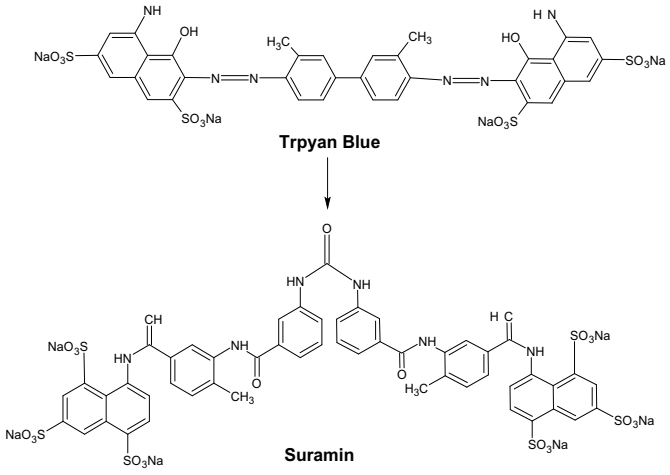


Fig 2. Chemical structure of Trypan Blue and Suramin.

Pentamidine

Pentamidine is the first choice of drug for the treatment of early stage *T.b. gambiense* infections. Pentamidine is an aromatic diamidine which accumulates in millimolar concentration in parasites and reduces trypanosome mitochondrial membrane potential[53]. It has been shown that pentamidine is transported by at least three different transporters[54]. 50% of the pentamidine influx is mediated through P2 (*TbAT1* gene) aminotransporter[55,56], a major transporter for adenosine and arsenic drugs. The rest is taken up by low capacity **h**igh **a**ffinity **p**entamidine **t**ransporter (HAPT1) and high capacity **l**ow **a**ffinity **p**entamidine **t**ransporter (LAPT1)[54]. Due to the use of different HAPT1 and LAPT1 transporters, melarsoprol resistance selected parasites that lack P2 are still sensitive to pentamidine[57,58]. Parasites selected for pentamidine resistance also showed a loss of virulence indicating resistance was associated with a fitness cost[59,60]. Hence propagation of resistance to pentamidine in the field is unlikely. When pentamidine resistance was selected in P2 knock out parasites, loss of virulence and a defect in HAPT1gene function was also observed[61].

The details of transporters involved in uptake and accumulation of pentamidine in the parasite are well characterized, but definite intracellular target was not determined[62] until recent investigations with the pentamidine analogue DB75 pinned down mitochondria as the target[53]. DB75 has shown to rapidly accumulate within the mitochondria of living trypanosomes and cause decrease in mitochondrial potential[53]. When accumulated in mitochondria, DB75 can inhibit whole cell respiration[53]. Several new pentamidine analogues, DB75 and DB820 have shown to be more potent and less toxic than pentamidine in murine models[63]. Recently, the prodrug DB289 (pafuramidine) was evaluated in a clinical trial and is currently in consideration for oral treatment in first stages of HAT[63].

Pentamidine is given intramuscularly and is administered for 7days with an interval of 24hrs. Although used for treatment of early stages, pentamidine has some activity in the CSF[64]. Low levels of pentamidine below one percent of plasma levels have been measured in CSF[65], hence ineffective for treating late stages. Pain at the site of injection, transient swelling, abdominal pain, gastrointestinal problems and hypoglycemia are the frequently reported side effects.

Summary of Pentamidine

Treatment: Only for early stages.

Limitations: Cannot cross BBB.

Mode of action: Binds to nucleic acid and targets mitochondrial membrane potential[53].

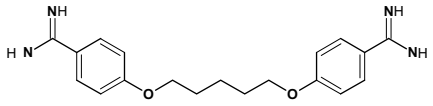
Resistance in field: No resistance observed in the field, resistance in laboratory strains is observed with mutations or loss of transporters associated with fitness cost.

Uptake: Transporters HAPT1, LAPT1 and P2 (*TbAT1* gene)[52,54].

Dosing: Intramuscular, 4 mg/kg bodyweight at 24hrs interval for 7days[13].

Side effects: Hypoglycemia, pain at the site of injection, diarrhea, nausea, and vomiting[13]

Structure:



pentamidine

Fig 3. Chemical structure of Pentamidine

1.5.2 Drugs used for late stages

Melarsoprol

Melarsoprol is an organic arsenic derivative, which is a potent trypanocidal drug effective against both human subspecies of trypanosomes: *T.b. gambiense & T.b.rhodesiense.* Melarsoprol is the most widely used drug for the treatment of late stage HAT despite extreme toxic effects[35,66]. 5-10% of the patients develop severe reactive encephalopathy and is lethal for half of them[32,66,67]. Melarsoprol remains the only drug available for the treatment of late stage infections caused by *T.b. rhodesiense.* Melarsoprol is also used for treating *T.b. gambiense,* when eflornithine is not available or affordable. Melarsoprol is rapidly converted to melarsen oxide upon administration (Fig.4). Trypanosomes are rapidly lysed when incubated with melarsoprol, but the mode of action behind lysis is not known. Melarsen oxide can interact with many sulfhydryl group-containing low molecular weight thiols in parasite and host leading to toxicity[68,69].

Development of resistance towards melarsoprol treatment and less sensitive isolates are observed in the field. Several factors (i.e. ATP binding cassette (ABC) transporters)[70]

are associated with the development of resistance towards melarsoprol but the major evidence is directed towards a reduced uptake of the drug by P2 amino transporter[44–47]. However, P2 knockout trypanosomes are only moderately sensitive to melarsoprol than wild type trypanosomes[58], indicating other possible routes of uptake of the drug. The other possible route of uptake might be through HAPT1[54].

Melarsoprol is given with a standard 10 day course with 2.2 mg/kg once per day[66,74]. The active form of melarsoprol (melarsen oxide) accumulates to a maximum of 1-2%(0-0.1μg/ml) in plasma and transverse the BBB. These concentrations are enough to clear the parasites[75]. As indicated above, side effects are severe and include reactive encephalopathy seen in 5-10% of the treated patients. Pyrexia, thrombocytopenia, headache, and pruritus are other frequently reported side effects[13].

Summary of Melarsoprol

Treatment: For late stages

Mode of action: Not known, multiple targets (possible mechanism is loss of ATP due to inhibition of glycolysis[76], and forms complex with trypanothione (MelT) and interfere with the trypanothione pathway[68])

Resistance in field: Observed field isolates which are less sensitive to Melarsoprol

Uptake: *TbAT1* (P2) and HAPT1

Dosing: Intravenous, standard 10 day course with 2.2 mg/kg once a day

Side effects: Encephalopathic syndromes, pyrexia, thrombocytopenia, headache, pruritus are the most frequent adverse effects.

Structure:

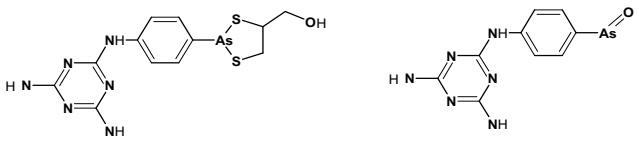


Fig 4. Chemical structure of Melarsoprol and its active form Melarsen oxide.

Eflornithine

Eflornithine or D,L-α difluoromethylornithine (DFMO) is an analogue of amino acid ornithine, which is an inhibitor of the polyamine biosynthetic enzyme ornithine decarboxylase (ODC)[77,78]. In most organisms, ODC is the rate-limiting enzyme for the synthesis of polyamines from ornithine. Eflornithine has similar affinities for mammalian and trypanosome ODC, but eflornithine is effective in parasites due to the

slower turnover of ODC enzyme (the half life of ODC in *T. b. gambiense* is about 18hrs[79]) than that of mammalian cells. Thus, treatment with eflornithine in trypanosomes depletes the net polyamine synthesis for a longer time compared to mammalian cells. Irreversible inhibition of ODC by DFMO and slower turnover of the enzyme leads to accumulation of S-adenosyl methionine (SAM) in parasites. Methyl group of methionine in SAM is chemically reactive and involved in transmethylation reactions. Depletion of ODC leads to inappropriate methylation of proteins, nucleic acid, lipids and other cell components[80]. Treatment with eflornithine leads to growth arrest and transformation of parasites into non-proliferating stumpy forms which are later cleared by the host immune responses[81]. Eflornithine is ineffective for *T.b. rhodesiense* subspecies due faster ODC turnover, which has a tentative half life about 4.3hrs[79].

Uptake of DFMO in parasites has been suggested to mainly occur by passive diffusion[82]. It was reported that an amino transporter gene, TbAT6 was deleted in eflornithine resistance selected trypanosomes[83]. These findings indicate that the uptake of eflornithine can be mediated by combination of passive diffusion and active selective transport.

Eflornithine is given intravenously with 100 mg/kg body weight at 6 hrs interval for a period of 14 days. The mean half life of eflornithine in plasma after intravenous administration is 3hrs, and 80% of the eflornithine administered is excreted in urine unchanged after 24hrs, hence large doses of prolonged intravenous doses must be given[84,85]. Fever, headache, hypertension, and gastrointestinal problems including diarrhea are the frequently reported side effects.

Summary of Eflornithine

Treatment: For late stages of *T.b. gambiense* infections.

Mode of action: Inhibition of Polyamine biosynthetic enzyme Ornithine decarboxylase (ODC)

Resistance in field: Not observed

Uptake: Passive diffusion across the plasma membrane[82]

Dosing: Intravenous, 100mg/kg body weight at 6hrs interval (which is equivalent to 400mg/kg per day), for a period of 14days

Side effects: Fever, headache, hypertension, convulsions, leucopenia, thrombocytopenia, and gastrointestinal problems including diarrhea[13].

Structure:

Ornithine **Eflornithine, DFMO**

Fig 5. Chemical structure of ornithine and its analogue Eflornithine

Nifurtimox-Eflornithine Combination chemotherapy (NECT)

Nifurtimox is a nitroheterocycle prodrug used in the clinic for the treatment of Chagas' disease also called American trypanosomiasis. Nifurtimox is reduced by parasite type I nitroreductases to generate cytotoxic nitrile metabolites[86](Fig.6). These generated nitrile free radicals may interact with cellular constituents in the parasite to cause cell death. Nifurtimox has the ability to penetrate through the BBB, and has half the plasma concentrations in the brain [87]. Nifurtimox monotherapy is not efficacious as *in vitro* IC_{50} of nifurtimox has a quite high IC_{50} (around 5μM) as compared to melarsoprol (10nM)[88,89]. Despite having less toxicity for parasites, nifurtimox is a clinically approved drug, can be given orally and can cross the BBB.

The combination chemotherapy of nifurtimox and eflornithine has reduced the dosage and treatment time of eflornithine monotherapy from an infusion every 6hrs for 14 days to 12 h for 7 days. The combination is given as intravenous eflornithine (400 mg/kg per day, every 12 hrs) for 7 days with oral nifurtimox (15 mg/kg per day, every 8 hrs) for 10 days. Efficacy of NECT is similar to eflornithine monotherapy[90].

With the frequent treatment failures due to poor patient adherence[91], the use of combinational therapy results in synergism, reducing dosages and also diminish probability of generating drug resistant mutants in the field.

Summary of NECT

Treatment: Combination therapy for late stages

Mode of action: Generation of reactive nitrile intermediates resulting in oxidative stress

Resistance in field: Not observed

Uptake: Nifurtimox is probably transported by purine amino transporter P2[92,93]

Dosing: Combination is given as intravenous eflornithine (400 mg/kg per day, every 12 hrs) for 7 days with oral nifurtimox (15 mg/kg per day, every 8 hrs) for 10 days

Side effects: Nausea, vomiting, anorexia and tremors are frequently reported[43].

Structure:

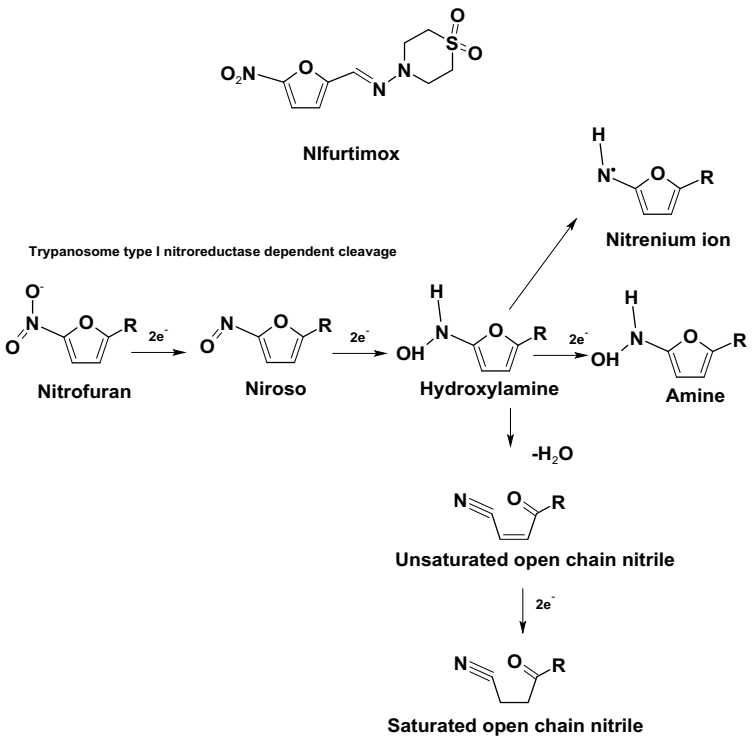


Fig 6.Chemical structure of Nifurtimox and sequential generation of reactive nitrile intermediates (nitrenium ion, saturated and unsaturated open chain nitriles) by type I nitroreductases in the parasite. Adapted with changes from Hall *et al*[86].

Clinical candidates

Fexinidazole

Fexinidazole (5-nitroimidazole Fig.7) is a new oral candidate in clinical development for the treatment of HAT. Nitroimidazoles (e.g. Megazol) are pharmacologically known anti trypanocidals inducing DNA damage in trypanosomes. A systemic drug screening of more than 700 nitro heterocyclic (mostly nitroimidazoles) assessing their parasiticidal activity led to the identification of fexinidazole[94]. When administered *in vivo* fexinidazole rapidly forms fexinidazole sulfoxide and fexinidazole sulfone (Fig.7)[95]. *In vitro,* fexinidazole principal metabolites are shown to be potent trypanocidal compounds and are active against both human pathogenic subspecies of *T. brucei*. When given orally fexinidazole is effective for both early and late stage murine

HAT models. The recent preclinical profile indicated that fexinidazole is safe and effective oral drug candidate that could be further evaluated for the treatment of HAT[95].

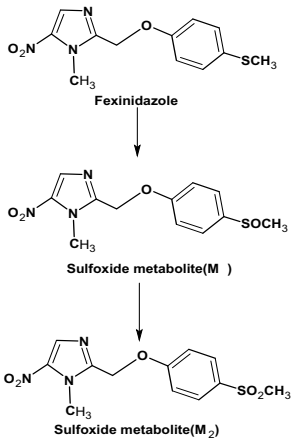


Fig 7. Chemical strexinidazole and its two metabolites. Adapted fromTorreele Els *et al*. [95]

SCYX-7158

SCYX-7158 (Fig.8) is an orally active benzoxaborole drug currently under development for the treatment of late stage HAT. SCYX-7158 is an optimized analog that was identified in a drug discovery project employing integrated biological screening, medicinal chemistry and pharmacokinetic characterization[96,97]. SCYX-7158 is trypanocidal against both human pathogenic subspecies of *T.brucei* and is efficacious in curing both stages of murine HAT models. Treatment is also efficient when given orally with low doses of 12.5 mg/kg (once a day for 7 days)[97]. *In vivo* pharmacokinetics on non-human primates showed that SCYX-7158 has a high bioavailability, low plasma clearance, and a 24 hr half-life with good tissue distribution. With promising preclinical profile, SCYX-7158 progressed into Phase I clinical trials in 2011[97].

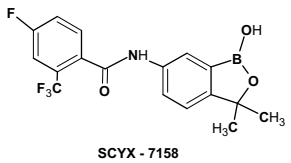


Fig 8. Chemical structure of Adapted from Jacobs *et al*. [97]

1.6 Role of transporters

1.6.1 The P2 Amino Purine transporter

Trypanosomes have broad range of transporters, which mediate the uptake of metabolites required for their survival. The toxicity of the drug is completely dependent on its uptake by transporters and the amount of drug retained intracellularly. It has been demonstrated that trypanosomes are less sensitive to melarsoprol when incubated in the presence of adenine and adenosine[73]. This indicated that melarsoprol transport is mediated through nucleobase/nucleoside transporter(s). The nucleoside transporter involved has been identified as P2, encoded by the *TbAT1* gene[72]. Some of the drugs (e.g. melarsoprol and pentamidine) which are used in clinic are exclusively transported through P2, hence loss of P2 transporter results in significant tolerance to these compounds[58,71,72]. It is also confirmed that melarsoprol resistant trypanosomes have lost or showed non silent mutations in the P2 gene[72,73,98]. In addition to P2 transporter, there are also other adenosine transporters named P1 which have broader affinity for many purines, but does not mediate the transport of melarsoprol[58,73]. Unlike other trypanosomatids (*i.e. Leishmania sp., T. cruzi*)[99,100] and most mammalian nucleoside transporters[101], *T.brucei* P2 has a selective affinity for adenine/adenosine and does not transport other purines and pyrimidines[73].

It has been shown that the uptake of adenosine is inhibited when incubated in the presence of pentamidine, indicating that the pentamidine uptake is also mediated through the P2 transporter[54,55]. Pentamidine rapidly accumulates forming intracellular millimolar concentrations, but pentamidine accumulation drastically drops in melarsoprol resistant trypanosomes *in vitro* and *in vivo* indicating P2 involvement[54,55]. It has been reported that 50-70% of pentamidine is transported by a putative adenosine sensitive pentamidine transporter (ASPT1)[54]. A decreased influx of pentamidine in *TbAT1*$^{-/-}$ trypanosomes demonstrated that *TbAT1* encodes the reported ASPT1 activity[54,58]. Involvement of P2 transporter for the uptake of melarsoprol and pentamidine is also supported with the reports of cross resistance[102,103]. P2 transporter has selective affinity for adenine /adenosine, but has the ability to transport diverse structures like melarsoprol and pentamidine. The reason for such transport was resolved with the elucidation of substrate recognition motifs of P1 and P2 transporters[104]. Despite having similar affinity for adenosine, P1 and P2 transporters have unique ways of recognizing these substrates. P1 forms two hydrogen bonds with the hydroxyl groups of the ribose, and N3 and N7 of the

adenine purine ring forms potential hydrogen bond donors or acceptors[104]. Instead, P2 makes its primary interactions with the N1(1-position) and NH2(6-position) of purine and does not involve any interaction with its ribose.

Further, the high affinity for adenosine is due to π-π interactions of purine ring and aromatic residues in the receptor, and electrostatic interaction of 9-nitrogen in the purine[104] (Fig.9a). Due to the presence of common functional groups like amidines in melarsoprol, pentamidine and adenosine, that attributes to resistance and cross-resistance [104] (Fig.9b).

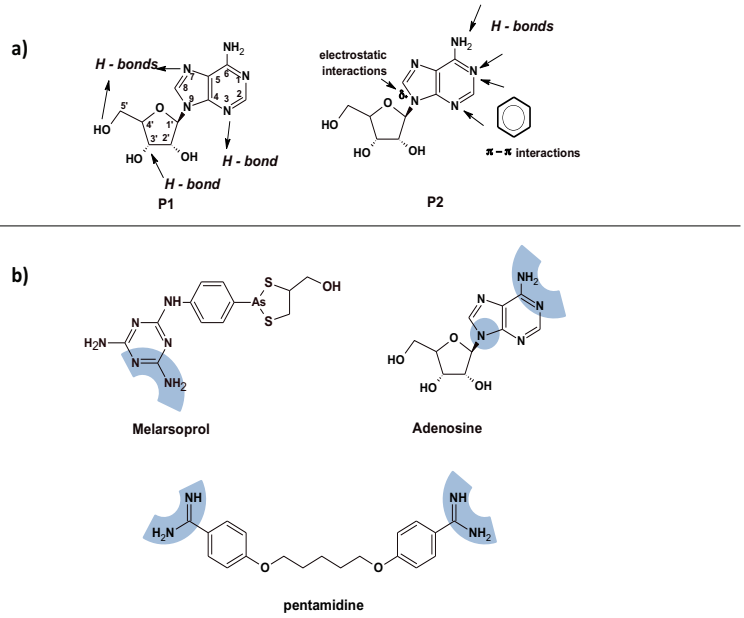


Fig 9 **a)** Substrate interaction of adenosine with P1 receptor (interactions of adenosine in the binding pocket N3, N7, 3'-OH, 5'-OH groups are indicated with an arrow), P2 receptor (N1, C6-NH2, Electrostatic interaction of 9th Nitrogen and π - π interaction of the purine ring and binding pocket are indicated with an arrrow[57,104]) Adapted with changes from Koning *et al*[57]. **b)** Amidine moiety's of melarsoprol, pentamidine which are like adenosine are represented in blue. Adapted with changes from Mäser *et al*[105].

1.6.2 The high affinity pentamidine transporter (HAPT1) and the low affinity pentamidine transporter (LAPT1)

The inhibition of adenosine uptake by pentamidine proved that pentamidine is transported through P2 transporter (K_m=0.26μM)[54,56]. However, *TbAT1*$^{-/-}$ null trypanosomes only showed two fold resistance to pentamidine when compared with wild type controls, indicating alternative routes of transport[58]. Alternative routes for the transport of pentamidine have been identified to involve a high affinity pentamidine transporter (HAPT1, K_m =36nM) and a low affinity pentamidine transporter (LAPT1 K_m=56μM)[54]. It is shown that loss of the P2 transporter results in resistance to some analogues of pentamidine (e.g. diminazene, propamidine, stilbamidine), but only low levels of resistance to pentamidine, indicating that pentamidine is transported through HAPT1and LAPT1. This explains the reason why resistance to diminazene (a drug used in veterinary medicine) is more common in the fields than that of pentamidine[54,56,58]. Melarsoprol might also be transported through HAPT1, due to slow lysis of *TbAT1*$^{-/-}$ compared with wild type trypanosomes in presence of this drug[58].

1.6.3 Other Purine transporters

The *T.brucei* genome revealed that trypanosomes have clusters of genes encoding members of equilibrative nucleoside transporters (ENT) family[106,107]. Besides the P1 and P2 purine transporters, trypanosomes possess a family of TbNT transporters (that encode P1 like activity) for the uptake of purines[108]. A cluster of six genes encoding nucleoside/nucleobase transporters (TbNT2/927, TbNT3, TbNT4, TbNT5, TbNT6, and TbNT7) with amino acid sequences similar to each other, that mediate the uptake of purines/ pyrimidines were identified[109]. It was shown that TbNT2, TbNT5, TbNT6, and TbNT7 are high affinity adenosine/inosine transporters and TbNT5 and to some extent TbNT6, TbNT7 mediate hypoxanthine transport[109]. The TbNT2 cluster of genes compensated purine transport in the absence of P2 transporter[110].

1.6.4 Efflux pumps

Alike mammalian cells, trypanosomes possess efflux pumps to excrete xenobiotic and unwanted metabolites[111]. The ABC transporters are conserved proteins, present in prokaryotes and eukaryotes that play a major role in drug efflux. ABC transporters are membrane proteins, which are coupled with ATPase activity, actively transporting

substrates against a concentration gradient[112]. Each ABC transporter is relatively specific for a given substrate[112]. For example the multi drug resistance protein (MRP) family extrudes a variety of organic anions[113] and p-glycoproteins efflux antibiotics, and most anticancer drugs. The mammalian MRPA and MRPE components of the MRP family have the ability to efflux drugs conjugated to glutathione[113]. Likewise, it has been speculated that trypanosomes also extrude conjugates of trypanothione through TbMRPA and TbMRPE transporters. Although an increase expression of TbMRPA is not seen in trypanosome drug-resistant isolates, the overexpression of TbMRPA in trypanosomes resulted in 10-fold increased resistance to melarsoprol[70]. Accordingly, depletion of TbMRPA made trypanosomes moderately hypersensitive to melarsoprol and melarsen oxide[114].

The overexpression of TbMRPE results in a two-three fold resistance with suramin[70]. These findings indicate that drug resistance could result from a combination of events including a lower uptake or reduced accumulation due to increased efflux of drugs.

Overview of the transporters involved in the drug uptake

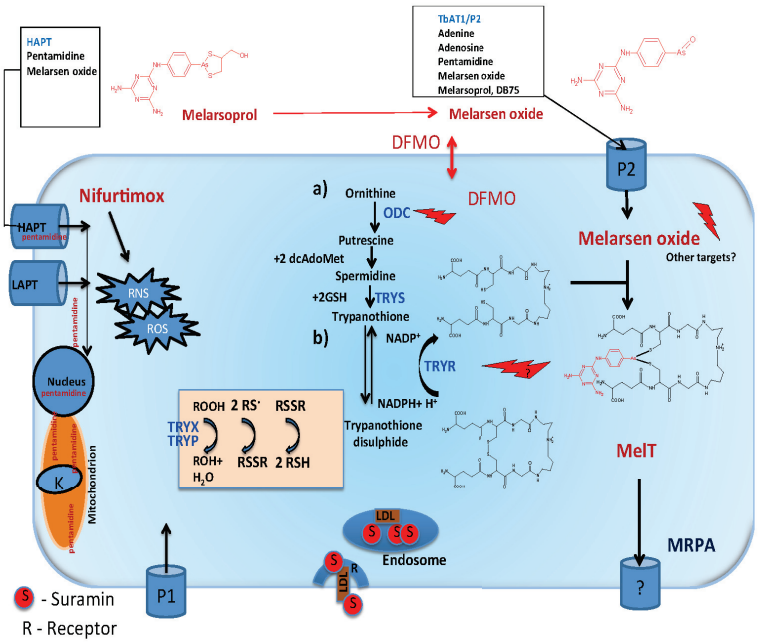


Fig 10. Overview of the transporters involved in the drug uptake, drug efflux, possible drug targets and mechanism of action in *T.brucei*. **a) DFMO** which enter into parasites by passive diffusion and targets Polyamine biosynthetic pathway enzyme ODC leading depletion of trypanothione levels **b)** Trypanothione metabolism is involved in quenching of ROS species by TRYX and TRYP. **1) Melarsen oxide** enters via P2 transporter and forms complex with trypanothione (MelT), which might inhibit TryR (trypanothione reductase regenerates trypanothione and maintains thiol-redox balance) resulting in accumulation of ROS species, and might also have other targets (mode of action is not definite). **2) Pentamidine** has the possibility of entering via P2, HAPT1, and LAPT1. Pentamidine binds to negatively charged components such as DNA(kDNA). **3) Suramin** when bound to LDL gets endocytosed and gets into endosome compartment or released into the cytosol (mode of action by suramin is not definite). **4)** It is speculated that **Nifurtimox** enter via P2 and generates nitrogen free radicals upon cleavage by parasite type I nitroreductases. MRPA transporters have the possibility to efflux the MelT conjugates **Abbreviations**: **DFMO**, DL-a-difluoromethylornithine (eflornithine); **MRPA**, multidrug resistance protein; **ODC**, ornithine decarboxylase; **TRYP**, tryparedoxin peroxidase; **2GSH**,glutathione; **TRYR**, trypanothione reductase; **TRYS**, trypanothione synthetase; **TRYX**, tryparedoxin; **(ROOH)** hydrogen peroxides, freeradicals **(RS·)** and disulphides **(RSSR)**; **RNS**, reactive nitrogen species; **ROS**, reactive oxygen species**;** **HAPT**, high affinity pentamidine transporter;**LAPT**,low affinity pentamidine transporter; S- suramin; **R**-receptor; **K**-kinetoplast; Arrows indicates active transport and double edged arrow indicate passive diffusion. Adapted with changes from Koning[71] & Fairlamb[115].

1.7 Novel targets for the development of drugs selective to parasites

1.7.1 Polyamine pathway

Ornithine decarboxylase

Polyamines are the organic cations which are derived from amino acids and present in all organisms[116,117]. Polyamine metabolites are essential for cell growth, free radical scavenging and tissue repair[117]. Production of putrescine from ornithine is by phosphate dependent ornithine decarboxylase (ODC) (Fig.11) is the first rate-limiting step in polyamine pathway. ODC is tightly regulated and has a short half life (10-30 minutes in mammalian cells), but ODC of trypanosomes are very stable, have a long half life and a slow turnover[79,118,119]. Drugs targeting ODC are effective against trypanosomes due to prolonged and effective depletion of polyamine metabolites, which are required for parasite proliferation and survival. As indicated above, DFMO is an inhibitor of ODC. ODC inhibition also results in the depletion of putrescine, spermidine, trypanothione and in the accumulation of ornithine, s-adenosylmethionine (AdoMet) and decarboxylated s-adenosylmethionine (dc-AdoMEt, Fig.11)[80,120]. These results indicate that metabolic differences in the polyamine pathway of host and parasites are targets for the development of new drugs against HAT.

S-adenosyl –L-methionine decarboxylase

The second rate-limiting step in the polyamine pathway is the generation of methylthioadenosine (MTA) from AdoMet (S-adenosyl–L-methionine) by the AdoMet decarboxylase (Fig.11). Like ODC, AdoMet decarboxylase is also a tightly regulated enzyme. In mammalian cells, MTA is produced from spermidine by aminopropyl transferase, spermidine synthase and spermine synthase[119]. Whereas in trypanosomes only the spermidine producing enzyme is active, the production of MTA is solely dependent on AdoMet decarboxylase[78,121]. It has been shown that irreversible inhibition of AdoMet decarboxylase with MDL73811 (Fig.11) in parasites cures *T. b. brucei* and drug resistant *T. b. rhodesiense* in mice[122]. Treatment with MDL7381 resulted in the accumulation of putrescine and low levels of spermidine, after rapid inhibition of AdoMet decarboxylase[122–124]. The half-life of MDL7381 was approximately 10 min[122]. Further advances were made by synthesizing a stable analogue Genz-644131, with better pharmacokinetics than MDL7381. However Genz-644131 was not efficient in curing late stage infections, but cleared parasites from blood stream and increased the survival rate[125]. MDL7381 and Genz-644131 are analogues of S-adenosyl –L-methionine which

do not compete with adenosine transporter for uptake, indicating transport routes other than P2[126]. Parasites have a unique S-adenosylmethionine transporter, and thus S-adenosylmethionine analogues accumulate selectively in parasites compared to mammalian cells[126–128]. With novels routes of transport and selective inhibition in parasites, development of these drugs might lead to a new chemotherapeutic approach for HAT.

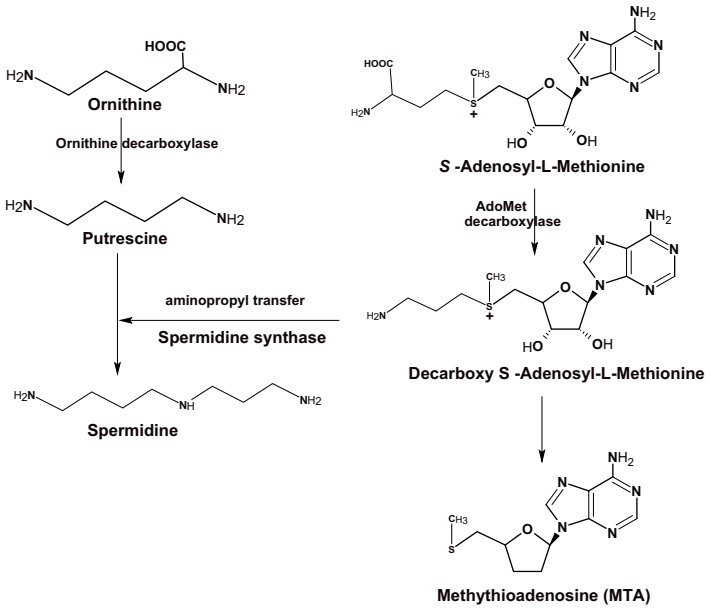


Fig 11. Rate limiting steps in in polyamine pathway for the production of spermidine. Adapted with changes from Bitonti *et al*[122].

MTA analogues

MTA is cleaved by specific MTA phosphorylase to adenine and methylthioribose-1-phosphate (MTRP)[129](Fig.12). Adenine is converted to ATP in the purine salvage pathway. Methionine is recycled from MTRP in a five step pathway which is one of the major route to salvage methionine in eukaryotes and prokaryotes[130–132]. MTA analogues with modifications on the ribose can be cleaved by MTA phosphorylase and form toxic analogues of MTRP[121]. Analogues of MTA (e.g. HETA, 5'-deoxy-5'-(hydroxyethylthio) adenosine) have shown to be trypanocidal and are efficient in curing *T.b. brucei* and *T.b. rhodesiense* infections in mice[133].

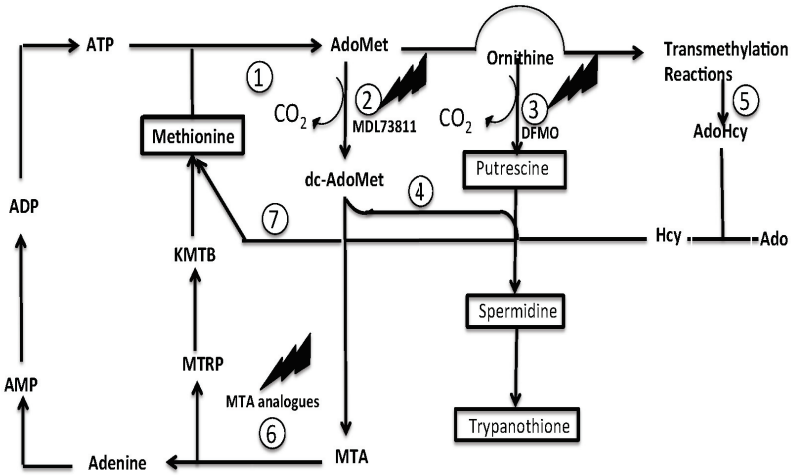


Fig 12:Overview of the Polyamine pathway and the Key enzymes: (1) AdoMet synthetase; (2) AdoMet decarboxylase; (3) ornithine decarboxylase; (4) spermidine synthase; (5) AdoHcy hydrolase; (6) MTA phosphorylase; and (7) 5-methyl-tetrahydrofolate methyltransferase. Abbreviations: **MTA**, Methylthioadenosine; **MTRP**, methylthioribose-1-phosphate; **KMTB**, ketomethylthiobutyrate; **AdoMet**, S-adenosylmethionine. Polyamine enzymes, which can be targeted by drugs are represented by black shaded inhibiting arrows. Adapted with changes from Bacchi *et al*[121].

1.7.2 Trypanothione pathway

Synthesis of trypanothione and subsequent reduction

Trypanothione is a product of the polyamine pathway, which is unique to trypanosomatids. A high concentration of at least one low molecular weight thiol is present in all organisms for the maintenance of intracellular reducing environment[134]. In mammals, glutathione is the key protein in maintaining a reducing milieu, but some organisms have analogues of glutathione[135,136]. In trypanosomes, trypanothione is the main low molecular weight thiol, which maintains the intracellular reducing environment. Conjugation of 2 glutathione molecules and one spermidine by bifunctional glutathionylspermidine/trypanothione synthetase (TryS) is the main event in the formation of trypanothione in trypanosomes (Fig.13), which is absent in mammalian cells[134]. The amount of trypanothione levels in the parasites is dependent on the availability of spermidine. Inhibition of enzymes like ODC that make spermidine or depletion of trypanothione synthetase mRNA leads to decrease of trypanothione and

accumulation of glutathione (GSH)[120,137,138]. Drugs targeting trypanothione synthetase or TryS down-regulated trypanosomes showed increased sensitivity to hydrogen peroxides[137]. These findings indicate that trypanothione play a major role in defense against oxidative stress[137,139].

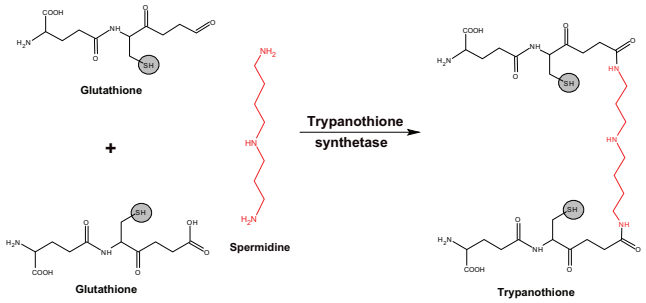


Fig 13. Formation of trypanothione by conjugation of two glutathione's with one spermidine by the enzyme trypanothione synthetase. Adapted with changes from Krauth siegel *et al*[140].

The sequence of redox events starts by recycling trypanothione to the thiol state $T(SH)_2$ from $T(S)_2$ by the flavoenzyme trypanothione reductase (TryR) at the expense of NADPH[134]. The physical and chemical properties of TR are similar to mammalian glutathione reductase (GR), the eukaryotic thioredoxin reductase (TrxRs) and lipoamide dehydrogenase[141]. Trypanosomes lack GR and TrxR hence TR is the sole enzyme linking reducing equivalents to trypanothione. The conditional knockout of TR in blood stream forms of *T.brucei* causes remarkable growth defects, due to the reduced turnover of TS_2, which impairs the reduction of tryparedoxin (TXN)[142]. Downstream of trypanothione, tryparedoxin (TXN) is the next predominant low molecular weight thiol. TXN shares homology with the thioredoxin (Trxs) type proteins[143]. TXNs are oxidoreductases with many common functional features with glutaredoxins (Grxs) that reduce ribonucleotide reductase (RR) and different peroxidases. Trypanosomes genome encode Trxs and Grxs are transcribed in both procyclic and bloodstream forms but the protein could not be detected using western blot[144,145]. Trypanosomes contain two isoforms of TXN that localize in the cytosol and the mitochondria. Depletion of cytosolic TXN leads to accumulation of glutathione, glutathionylspermidine and trypanothione as a compensatory response and the susceptibility to external H_2O_2 was

significantly enhanced, indicating the direct role of TXN in defense against oxidative stress[146,147]. The elevated levels of other thiols and trypanothione do not counterbalance the impaired defense against oxidative stress.

$T(SH)_2$/TXN transfers reducing equivalents to peroxidases, enzymes involved the detoxification of peroxides. Unlike mammalian cells, trypanosomes lack catalases and selenocysteine containing glutathione peroxidases for the detoxification of hydrogen peroxides. Trypanosomes have glutathione peroxidase-type enzymes (Pxs) which differ from mammalian peroxidases by having cysteine residue in the active site replacing selenocysteine[148,149]. The defense against oxidative stress is solely dependent on the transfer of reducing equivalents over the cascade of trypanothione pathway TryR/$T(SH)_2$/TXN(Fig.14), to cytosolic and mitochondrial peroxidases[147]. Depletion of mitochondrial Pxs by RNAi does not have any phenotype, but depletion of cytosolic Pxs impairs cell growth and enhanced H_2O_2 sensitivity[147].

$T(SH)_2$/TXN also reduce RR which is a key enzyme for the biosynthesis of DNA precursors[150]. RR catalyzes the *de novo* synthesis of deoxyribonucleotides by reducing ribonucleotides[151]. Deoxyribonucleotides are the building blocks for replication and repair of DNA. Trypanosomes have a single mitochondrion, which contains large interlocked DNA rings known as kinetoplastid DNA (kDNA). UMSBP is a zinc finger protein involved in the initiation of replication and the segregation of kDNA minicircles [152]. The activation of UMSBP and binding to DNA is dependent on its redox state[152,153]. However there is little evidence suggesting that the redox regulation by TXN play a role in the replication of kDNA.

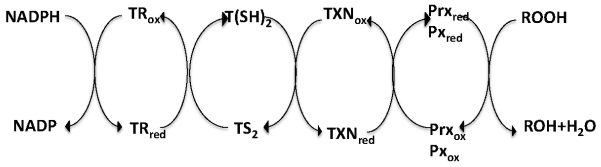


Fig 14. Redox cascade of trypanothione pathway in detoxification of hydroperoxides. Reducing equivalents are transferred to peroxidase (Px) from, TXN, $T(SH)_2$, TR, with the expense of NADPH as the primary electron source. Abbreviations: Tryparedoxin (TXN); Trypanothione [T(SH)2]; trypanothione reductase (TR); Px, peroxidase; ROOH ,hydroperoxides. Subscripts ox: oxidation, red: reduction, [$T(SH)_2$]-dithiol, TS_2 disulfide. Adapted with changes from Krauth-Siegel *et al*[140].

TryR, TryS and TXN are essential for the survival of parasites and their absence in mammalian cells, constitute a putative target for the development of new drugs d[145,154,155]. TryR is an homodimeric enzyme with a large, negatively charged, disulfide-containing substrate binding site[140]. Due to the large active site, a number of antimicrobial and antitumor drugs have been shown to inhibit TryR activity in a concentration- and time-dependent manner[156].

Several high throughput-screening (HTS) campaigns have been accomplished to identify inhibitors against TryR. None of them resulted in drugs that cure infection with *T.brucei* in mice [157–160]. Recently, a HTS was performed on a coupled system containing the whole trypanothione cascade. This HTS identified compounds that inhibited tryparedoxin and peroxidases coupled to trypanothione pathways [161]. These compounds were also cytotoxic for *T. brucei*. The efficacy of these compounds in curing *T.brucei* murine models is yet to be tested.

1.7.3 Purine metabolism

Purines play a vital role in all living organisms. Purines are constituents of nucleic acids, secondary messenger molecules and are involved in cellular metabolism. In general, purines are synthesized *de novo* or can be salvaged from the extracellular or intracellular milieu[162]. Trypanosomes possess an extensive network of purine salvage enzymes but cannot synthesize purines *de novo*[163]. Thus, these differences between host and parasite for meeting their purine requirement can be exploited in development of chemotherapy against parasite infections. Trypanosomes take up different purines through various transporters and convert them into essential nucleotides through salvage enzymes (Fig.15). However, due to the existence of multiple pathways and alternative transport systems, parasites can adapt to the absence or inhibition of a single enzyme and transporter[162]. For example, *T.brucei* high affinity transporter *TbAT1* mediates adenosine transport, but the deletion or loss of *TbAT1* does not confer a marked change in the phenotype or fitness cost[57,104,164]. In the same way, nucleotide synthesis of adenosine can be completed through either cleavage-dependent or independent high affinity adenosine kinase pathway. In the cleavage dependent pathway, adenosine is cleaved to adenine by a low affinity adenosine nucleoside hydrolase: the inosine adenosine guanosine

nucleoside hydrolase (IAG-NH) followed by the phosphoribosylation by adenine phosphoribosyltransferase (APRT)[162]. In the cleavage independent pathway, the phosphorylation to AMP and ADP is directly done by adenosine kinase (AK). The partial depletion of AK by RNAi confers resistance to adenosine analogues[165,166], but parasites otherwise survive, indicating an adaptation of parasites to the alternative salvage pathway. Thus, designing drugs that are selectively transported by parasites and have broad-spectrum inhibition of salvage enzymes is challenging. A combination of nucleoside analogues that have synergistic effect in affecting different components of the parasite purine metabolism might be a promising nucleoside analogue therapy for treating HAT.

Several nucleoside analogues have shown to display a remarkable cytotoxicity for *T. brucei in vitro*, and several have shown to cure the murine infection with *T. brucei*[166–169]. In this work, we have shown that the combination of cordycepin and deoxycoformycin cures the infection with both human pathogenic trypanosoma subspecies and is efficient in treating late stage infections[168,169].

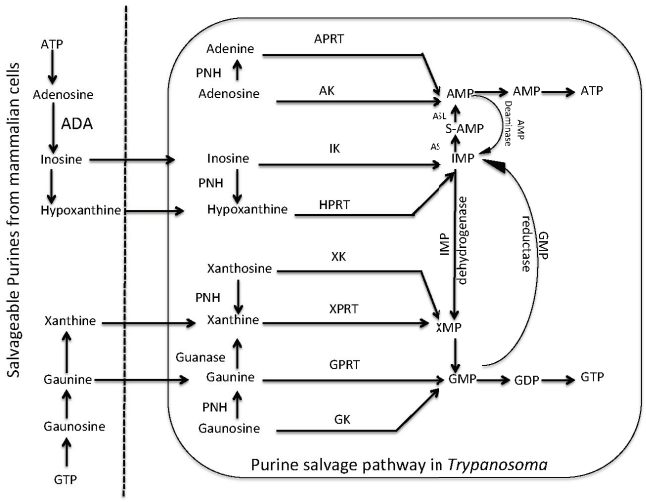


Fig 15. Purine salvage pathways in trypanosomes are shown inside the square. Purines that undergo degradation and can be salvaged from mammals is shown outside the square. In mammals ATP is dephosphorylated to adenosine, adenosine gets deaminated by adenosine deaminase (ADA) to inosine and gets phosphorylated to hypoxanthine. GTP is catabolized to guanosine, gets phosphorylated to guanine and oxidatively deaminates to xanthine. Trypanosomes can salvage all the dephosphorylated purines. Abbreviations and enzymes involved in salvage pathways: **PNH**, purine nucleoside hydrolase activities; **APRT**, adenine phosphoribosyltransferase activity; **AK**, adenosine kinase or phosphotransferase activity; AMP deaminase (EC 3.5.4.6); **ASS**, adenylosuccinate synthetase (EC 6.3.4.4); **ASL**, adenylosuccinate lyase (EC 4.3.2.2); **IK**, inosine kinase or phosphotransferase activity; **HPRT**, hypoxanthine phosphoribosyltransferase activity; **GK**, guanosine kinase or phosphotransferase activity; **GPRT**, guanine phosphoribosyltransferase activity; guanase (EC 3.5.4.3); **XPRT**, xanthine phosphoribosyltransferase activity; GS-**GMP synthetase** (EC 6.3.5.2); **GMP reductase** (EC 1.6.6.8); **IMP dehydrogenase** (EC 1.2.1.14); **XK,** xanthosine kinase or phosphotransferase activity, **AMP, ADP, ATP**(Adenosine mono , di and triphosphate forms); **GMP,GDP,GTP**(guanine mono, di and triphosphate forms).Adapted with changes from Fish *et al* & Carter *et al*[163,170].

1.8 Development of new drugs against HAT is needed.

Drugs involved in the current day treatment against HAT require parenteral administration, show difficulties in treatment regimen, poor efficacy, stimulate development of resistance and unacceptable toxicity related issues. Besides these complications, there is no single drug that can be used for treatment of both stages, or against both subspecies causing HAT. The effective drugs are few and with no

alternatives available, there is an urgent need for the development of new medicines, which are selective against parasites without toxic effects for mammalian cells or with a significant selectivity window.

Target-based High throughput screening (HTS)

The strategy to identify new lead compounds involves target screening ***in vitro*** followed by testing of anti-parasitic activity on whole parasites. Recent technological advancements in drug discovery projects, and the use of robotic liquid handling facilitates the high throughput screening (HTS) of large chemical libraries. HTS involves the identification of hits from the screen, which is followed by the optimization into a lead compound. HTS campaigns were performed using trypanothione reductase, hexokinase, N-myristoyl transferases, DNA topoisomerases, fatty acid biosynthesis and purine salvage enzymes as targets[161,171–173]. The lead compounds from these HTS have been identified as potent trypanocidal compounds, but further lead optimization is yet to be done to improve pharmacokinetics for testing in mice models.

Whole cell based assays

An alternative approach to identify leads when a target is not known is through whole cell growth inhibition assay. Validating trypanotoxic drugs in the screen has been measured using Alamar Blue (a redox indicator) or its variant WST-1 and by quantification of cellular ATP levels using a luciferase assay[168,169,174,175]. Every lead compound optimized in a drug screen has to be tested on whole cell trypanosome assay and other mammalian cells for differential toxicity. Testing the differential toxicity is the primary filter to avoid toxic drugs. HTS campaigns are fast and cost effective resulting in the development of large lead series for improving clinical success against HAT.

2. AIMS

The main aim of this thesis work is to develop new, cost-effective, safe and easily managed drugs for the treatment of African Trypanosomiasis.

Specifically:

1. We developed a real time imaging to monitor the efficacy of drugs to clear parasites in various organs using bioluminescent model.
2. We tested a nucleoside library consisting of 2,200 analogues for trypanocidal activity. We have selected cordycepin which when administered in combination with deoxycoformycin was effective as a treatment for late stage African trypanosomiasis.
3. We synthesized and characterized several deaminase resistant cordycepin analogues, which can be used as standalone drugs.
4. We performed a focused screen consisting of 5,500 lead like compounds to identify new drugs.
5. We discovered biochemically and functionally characterized trypanotoxic sulfur containing compound (ebsulfur), targeting trypanothione metabolism selectively in parasites.

3. MATERIALS AND METHODS

3.1 Parasite cultures

In vitro cultures were done using trypanosome stabilates, which are characterized as pleomorphic and monomorphic. For instance, *T.b. brucei* (AnTat1.1E) is a pleomorphic strain, which has two different morphological forms, long slender and short stumpy forms when cultured *in vitro*. This strain mimics the transitional forms that exist in the host. *T.b. brucei* strain 427 (s427; also known as MiTat 1.2/221 or BS 221), which are incapable of transforming into short stumpy forms, are called monomorphic strains. *TbAT1-/-* stabilate that lack P2 transporter were constructed in *T.b. brucei* strain 427. All the parasites were cultured in minimal essential medium (HMI-9) containing 10% of fetal bovine serum and serum plus supplement.

3.2 Whole cell proliferation assay using WST-1

In order to perform dose response curves and identify *T.brucei* proliferation inhibitors we employed WST-1 reagent as our readout. Metabolically active cells cleave WST-1 a tetrazolium salt (Pink in color) to yield soluble formazan (orange in color). Amount of formazan released directly corresponds to the number of viable cells. Formazan absorbance is spectrophotometrically monitored at 450nm.

In brief, suspensions of *T.brucei* were seeded in wells containing dilutions of drug solutions. Plates were incubated at 37°C and 5% CO_2 for 72hrs. For detection of cell viability, WST-1 reagent (Roche) was added to each well and the plates were incubated for additional 2hrs at 37°C and 5% CO_2. Absorbance was recorded from the plates using a multi-well spectrophotometer Victor2 (PerkinElmer) at an excitation wavelength of 450 nm.

3.3 Bioluminescent model and luciferase assay

To monitor *in vivo* dissemination of parasites and trypanocidal activity of drugs, we transfected *Renilla luciferase* (Rluc) gene into β-tubulin region of *T.brucei* parasites. The recombinant luciferase tagged parasites emit luminescence in the presence of its substrate coelenterazine. The amount of luminescence emitted by parasites corresponds to the number of viable parasites (Fig.15) since luciferase is constitutively expressed only in viable parasites.

Bioluminescence model

Bioluminescent model is a non-invasive method, which allows to follow the dynamics of infection and therapeutic efficacy of drugs in real time. To monitor spread of parasites in various organs and the efficacy of drugs for treatment of *T.brucei* infection *in vivo*, we infected mice with Rluc-tagged parasites. Luminescence was measured in real time by injecting mice with the substrate coelenterazine. Luminescence was measured in photons/ second/ cm2/ steradian (p/sec/cm2/sr) using an IVIS Imaging System 100 (Xenogen Life Sciences) and the Living Image H 2.20.1 software (Xenogen).

Trypanocidal assay

To measure trypanocidal activity of a given drug, serial dilutions of the drugs were incubated with $2.5*10^5$/200µl *T.b. brucei* (*Rluc*) in 96-well plates. Trypanocidal activity of the drug was monitored between 0 -10hr after initial drug exposure. Luminescence was measured 5min after addition of 10 µM of the substrate coelenterazine (Nanolight). Luminescence was quantified in a Microbeta Jet luminometer (PerkinElmer) in triplicate samples at each time point and drug dilution.

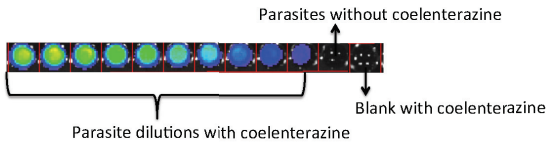


Fig 15. Luminescence emission from serial dilutions of Rluc parasites in the presence of coelenterazine. Parasites without coelenterazine, and medium in the presence of coelenterazine, serve as blank which do not emit any luminescence.

3.4 Focused screen for identification of trypanotoxic compounds on whole cell *T.brucei* assays.

A focused screen with WST-1 based assay was developed for the detection of compounds with potent anti proliferative effect against *T. brucei*. A focused library of 5,500 diverse compounds and 2,200 nucleoside analogues (Medivir chemical library) were screened for viability against *T.brucei* in a 96-well format. The compounds selected based on diversity represent a 80,000 small-molecule large library of the Laboratory for Chemical Biology at Karolinska Institutet (LCBKI). The hit to lead development was done by setting different filters to eliminate both non active compounds and generally toxic compounds.

Screening funnel

In Summary, primary hits with trypanotoxic activity <1μM were confirmed by determination of their IC_{50} in dose response curves using two viability/ proliferation assays: WST-1 and Alamar blue. A second filter was used to eliminate selected compounds from the primary hits that were toxic for the mammalian cell lines L-929 (ATCC-CCL-1) and MOLT4 (ATCC-CRL-1582). Compounds with high trypanocidal activity and acceptable selective window for mammalian cells, were thus selected, and the series of compounds that represent these were also tested. These series were clustered based on common scaffold and functional groups. The compound series with favorable predicted physiochemical characteristics, and feasibility of chemical synthesis were prioritized for further testing.

Lead compounds with high trypanocidal activity but moderate selectivity to mammalian cells, were optimized by studying the structural activity relationship (SAR) of several closely related analogues available in the chemical library of LCBKI. Optimized analogues were chemically synthesized, its activity confirmed *in vitro* and were tested in animal models of infection.

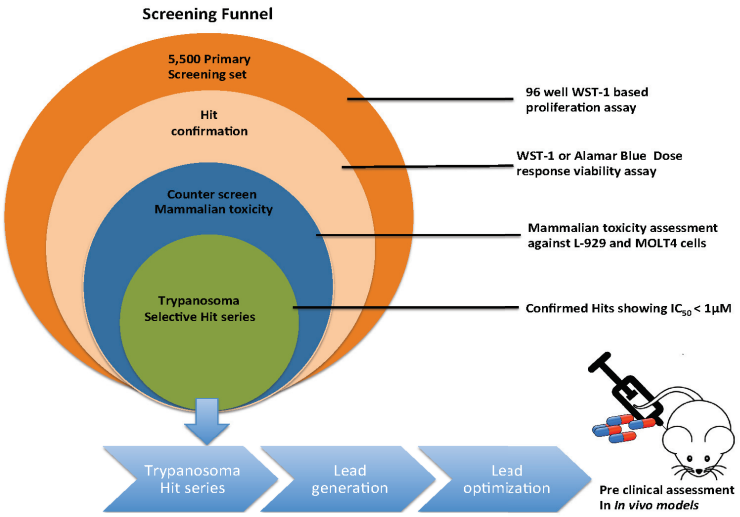


Fig 16. **Hit to lead development involved in HTS screening campaign.** Primary hit identification is assessed using WST-1 assay. **Filter 1**: To rule out false negatives resulting from colored compounds and other factors, alternative Alamar blue viability assay was used to confirm the hits. **Filter 2:**Assessing differential toxicity against mammalian cells eliminates drugs exhibiting general toxicity. **Filter 3:** Compounds showing good trypanotoxic activity and selectivity to mammalian cells were considered as lead compounds. Lead optimization was done using chemical synthesis and related analogues available in the chemical library. Efficacies of optimized compounds were later tested in trypanosomiasis mice model.

3.5 African Trypanosomiasis *in vivo* models:

Post exposure prophylactic (PEP) and acute infection models: To check if the compounds have the ability to prevent the infection, mice were infected intra-peritoneal (i.p.) with 2000 *T. b. brucei* (Antat1.1E) and 2hrs later treated with drugs dissolved in phosphate buffer saline (PBS). In the acute infection model, treatment was started 5 days post infection (dpi), when parasitemia was measurable in the blood smears. Untreated mice received equal volumes of PBS to that of treated. Parasitemia was determined in tail vein blood smears up to 90 days post treatment. Surviving mice without signs of parasitemia until 90 days were considered cured (Fig.17).

Late stage infection model: Mice infected i.p. with *T. b. brucei* (Antat1.1E) or *T. b. gambiense or rhodesiense* are treated i.p or oral gavage or subcutaneously starting from 21-day post infection (dpi). Immunostaining on brain sections of mice sacrificed on day 21 show distribution of parasites in parenchyma (Fig.18). Thus treatment starting after 21

days post infection serves as a good model to monitor efficacy of treatment in second stage. After treatment mice were monitored for weight loss and parasitemia for 90 days. Mice with negative parasitemia during 90 days were considered cured.

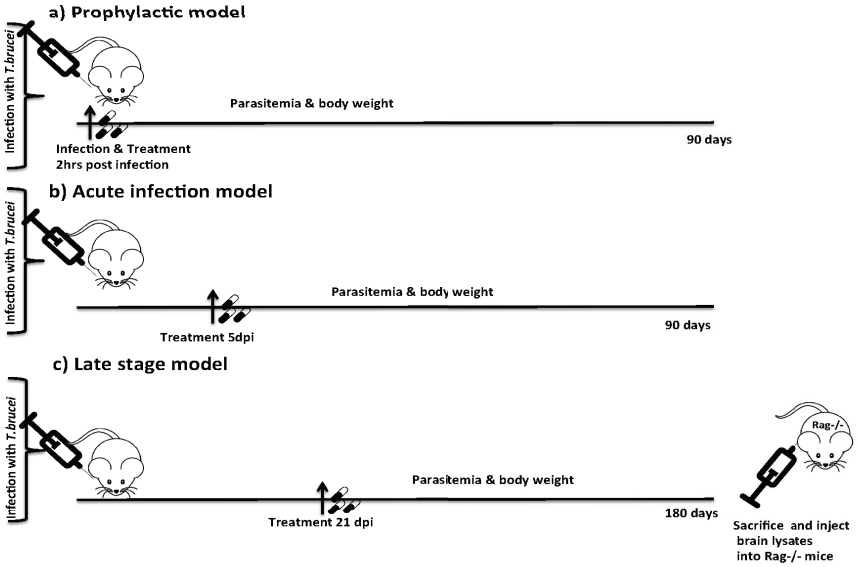


Fig 17. African trypanosomiasis *In vivo* models

Brains from *T.brucei* infected mice were collected and frozen on dry ice to examine the presence of parasites in the brain parenchyma. Frozen tissues were sectioned at the level where lateral ventricles containing choroid plexus, corpus callosum, and septal nuclei are visible. Sections were fixed and immunostained with anti VSG to visualize parasites (Red color) and anti glucose transporter-1(GLUT-1) for endothelial blood vessels (green color)[176].

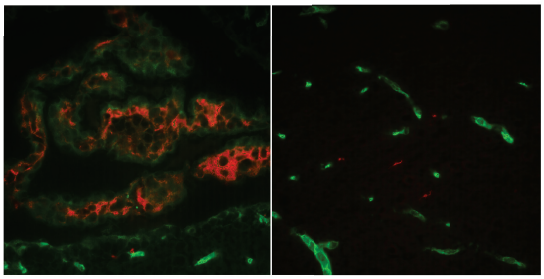


Fig 18.Immunostaining of 21dpi brain sections showing presence of parasites in choroid plexus and septal nuclei.

3.6 TryR activity

To study inhibitors targeting TryR, we used an assay in which reduction of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) added in the reaction buffer was monitored or measured the consumption of NADPH at 340nm (Fig.19). Thiol compounds like trypanothione when reduced will cleave the colourless DTNB to a yellow colour $2TNB^-$, which can be measured spectrophotometrically at 412nm. The reduction of trypanothione is completely dependent on the activity of TryR, hence inhibitors targeting TryR will result in low absorbance at 412nm (due to low levels of reduced trypanothione) or diminished consumption of NADPH[177–179].

TryR activity was assayed in the solution containing 50mM Tris-HCl pH 7.5, 200 μM NADPH, 1mM EDTA, 10 μM $T(SH)_2$ in the presence of 1mM DTNB. The absorbance at 412 nm or 340nm was followed in a VERSA microplatereader (Molecular Devices, Sunnyvale, CA, USA).

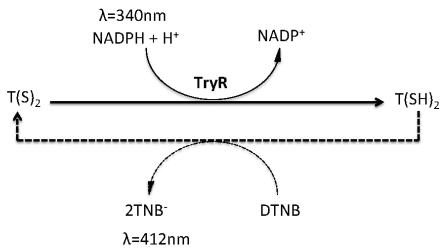


Fig 19. Activity of TryR can be monitored by reduction of DTNB (colorless) to $2TNB^-$ (Yellow color λ =412nM) or consumption of NADPH (λ =340nM). Adapted with changes from Jones *et al*[177].

4. RESULTS AND DISCUSSION

4.1 Monitoring dissemination of parasites and efficacy of drugs using real time *In vivo* bioluminescent model (Paper I)

In order to evaluate the dissemination of *T.brucei* infection and the therapeutic efficacy of drugs in murine models we constructed recombinant *Renilla* luciferase (Rluc) expressing *T. brucei*. We opted *Renilla* luciferase than firefly luciferase since it is shown that firefly luciferase impedes the trypanosomal growth by accumulating in glycosomes[180]. Moreover the substrate coelenterazine is less polar than D-luciferin, which can easily pass through cell membranes. The amount of luminescence emitted is proportional to the number of parasites (Figure 1D, Paper I). Thus luminescence by Rluc *T.brucei* can also be used in whole cell assay ***in vitro*** to evaluate trypanocidal activity of drugs (Figure 5C, Paper I).

The kinetics of infection and the distribution of parasites were monitored in murine models by infecting with luciferase transgenic monomorphic and pleomorphic strains followed by luminescence measurement with a biophotonic camera in real time after intraperitoneal inoculation of luciferase substrate. The pleomorphic strains allowed monitoring the development of infection over 30 days whereas monomorphic strains are more virulent resulting mortality of mice approximately 5 days after infection (Figure 2, Paper I). The method was validated by measuring luminescence and parasitemia in parallel. Luminescence was usually located in the peritoneal cavity, confirming that all mice are positive for parasitemia (Figure 2A, Paper I). However the intensity of luminescence was not always associated with parasitemia levels. The pattern of light emission was dependent on the type of substrate administration. Substrate when given intravenously, light emission was throughout the body (Figure 4B, Paper I) indicating that the distribution of coelenterazine was not homogenous.

During the course of infection we observed that parasites localized in the testis as early as 3 days post infection, suggesting a preferential testis tropism. Similar patterns of testis tropism were also observed in Rag-/- mice, indicating that the tropism of parasites towards testis is not due to the pressure posed by the immune responses (Figure 3A, Paper I).

Immunostaining of a testis section revealed that most of the parasites have crossed the blood testis barrier and accumulated under the basal lamina of seminiferous tubules (Figure 3B, Paper I). Parasites were not observed inside the seminiferous tubules,

confirming previously published data[181]. It has been reported that the presence of trypanosome in interstitial space of the testis results in endocrine imbalance, testicular lesions and diminished testosterone levels[182,183]. Supporting this data we never observed offspring generation when infected males were mated with uninfected females, suggesting male sterility.

We then tested if the *in vivo* bioluminescent infection models could be used to evaluate the outcome of treatment with a drug in real time. We observed that treatment with sub-curative doses of cordycepin and deoxycoformycin resulted in the reduction of parasitemia to non-detectable levels, but few days later reestablishment of the infection was apparent in the testis of mice (Figure 5D, Paper I). These results suggest that ability of the drug to kill parasites in testis is hampered due to the presence of blood testis barrier. Our data indicate that preferential testis tropism must be considered during drug development, since parasites might be protected from many drugs by the blood-testis barrier. However it must be noted that the current model uses intraperitoneal infection and substrate administration, which may somehow bias the observed dissemination of the parasites.

4.2 Preclinical assessment of cordycepin and deoxycoformycin combination therapy (Paper II)

Our group had previously shown that treatment with the adenosine analogue cordycepin (3'- deoxy adenosine-Cy) in combination with deoxycoformycin cured murine late stage models of African Trypanosomiasis[168].

We first attempted to find new nucleoside analogues, which are as trypanotoxic as cordycepin and if possible are deaminase resistant. For this purpose, a nucleoside library consisting of 2,200 analogues was screened on with a whole cell *T.brucei* viability assay using WST-1 reagent. The screen resulted in 14 primary hits, which are trypanotoxic less than 1μM. But, only four molecules showed differential toxicity for reference mammalian cells. Cordycepin, tubercidin (7-deazaadenosine) and two diamine molecules that are intermediates in the chemical synthesis of nucleosides showed anti-parasitic activity. Despite excellent levels of anti-parasitic activity, tubercidin was not considered for further studies since it has been shown that parasites rapidly adapt to the presence of this nucleoside. Moreover, host toxicity for tubercidin has been reported[162,184]. Hence, we focussed our further studies to develop and characterize cordycepin as our lead compound.

Oral, intra peritoneal and subcutaneous administration of cordycepin & deoxycoformycin results in complete cure of late stage African Trypanosomiasis in murine models.

Pentostatin (deoxycoformycin), a FDA approved drug, is an adenosine deaminase inhibitor and has been shown to enhance the biological activity of cordycepin[185]. The combination of cordycepin and pentostatin (deoxycoformycin-dCF) proceeded to phase I/II clinical trials for the treatment of refractory TdT^+ leukemia[186]. Thus, we chose to develop the combination of cordycepin and deoxycoformycin for the treatment of African Trypanosomiasis. The intravenous (i.v) inoculation of 0.25 mg/kg/ day deoxycoformycin resulted in inhibition of adenosine deaminase and had maximum tolerated dose (MTD) of up to 8mg/kg/day for 3 days in Beagle dogs[187]. We determined that 10 doses of 0.1 or 0.2 mg/kg/day dCF in combination with 2 mg/kg of cordycepin when given intra peritoneal (i.p) or subcutaneously were the minimal effective dose for curing late stage murine *T. b. brucei* infection (Figure 2 & Figure 4C, Paper II). The dose of deoxycoformycin required for treatment was far below the MTD. The combination was also effective in curing late stage murine infections with human pathogenic subspecies, *T. b. gambiense* and *T. b. rhodesiense* (Figure 5, Paper II). Weight loss was observed during the course of treatment with the combination but weight was completely regained after the treatment (Figure 2, Paper II). Since current day treatment is involved with difficulties in administration, we tested if combination of cordycepin and deoxycoformycin can be administered orally. When administered orally by gavage the combination was also effective in curing late stage infection if administered with a gastric proton pump inhibitor (omeprazole) (Figure 4 B, Paper II).

10% of the patients develop post treatment reactive encephalopathy (PTRE) when treated with arsenic compounds like melarsoprol[1,32,67]. PTRE can be studied in mice models by giving sub curative doses of Berenil. Sub curative doses of Berenil can eliminate trypanosomes in the blood circulation but not in the CNS, thus treatment with Berenil can be exploited in studying PTRE in mice[188]. It is reported that pro inflammatory cytokines m-RNA of both IL-6 and TNF-α was detected in the brains of mice developing post treatment reactive encephalopathy[188]. The treatment with cordycepin and deoxycoformycin diminished accumulation of pro-inflammatory cytokine m-RNA transcripts, indicating treatment ameliorates CNS related pathology (Figure 6, Paper II), a consequence of the interaction between the host immune system and the parasite infection [1,189].

Cordycepin induces programmed cell death in trypanosomes

Cordycepin activated the programmed cell death of *T. brucei*. Cordycepin treated parasites showed hypo diploid sub G1 peaks, DNA fragmentation and expression of phosphatidyl serine within 1hrs. These are typical characteristics of programmed cell death[190] (Figure7A;B;C , Paper II). A secondary necrosis of trypanosomes was observed after 7 hrs indicating that it follows the programmed cell death (Figure7D, Paper II). It has been reported that the trypanocidal effect of pentamidine and other diamidines is mediated by the collapse of mitochondrial potential[53], however no change in the mitochondrial redox potential was observed in cordycepin treated parasites.

Development of resistance to cordycepin is associated with fitness cost

The emergence of resistance to current drugs is one of the factors behind the treatment failures against many infections. Thus, we studied if trypanosomes could develop resistance to cordycepin. Using increasing sub-lethal cordycepin concentrations for a period of four months, we achieved 40-60 fold resistance in pleomorphic and monomorphic *T.b. brucei* stabilates (Figure 8, Paper II). The resistant trypanosomes were not cross-resistant to any of the current drugs used in the clinic suggesting there is no complete overlap in the targets and transporters used by cordycepin with those from the other drugs (Figure 8, Paper II). Cordycepin-resistant parasites showed slower growth *in vitro* and diminished virulence *in vivo* indicating that the development of resistance was associated with an important fitness cost for the parasite (Figure 9, Paper II).

Since resistance towards melarsoprol and pentamidine is associated with mutations or loss of the adenosine transporter *TbAT1*, we sequenced *TbAT1* along with other transporters and some of the purine salvage enzymes in cordycepin resistant trypanosomes. We did not observe any deleterious mutations in the selected genes but with exception of *TbAT1*. Insertion or deletions in *TbAT1* were detected in the pleomorphic strains and complete loss of *TbAT1* gene was observed in the monomorphic resistant parasite clones. However, *TbAT1* knockout parasites did not show increased resistance to cordycepin, at least in the same levels observed in resistant parasites, indicating that if required, *TbAT1* alone does not contribute to the accumulation of resistance (Figure 10, Paper II), and that its function in the uptake of the analogue could be compensated by other receptors.

Trypanosomes cannot synthesize purines de novo, and are completely dependent on the host for their purine uptake. Thus, the parasites host-dependent purine metabolism

represents a rational target for drug development. All together, besides having some contraindications related to dCF, we show that low doses of Cy & dCF combination could be a new orally administrable therapy for HAT.

4.3 Development of deaminase resistant cordycepin analogues (Paper III)

It is shown that deoxycoformycin (dCF) has lethal effect in the development of mouse embryos[191]. Since dCF has shown to be a teratogen, we aimed at developing dCF independent deaminase resistant cordycepin analogues that could be used in a monotherapy scheme in treatment of African trypanosomiasis. In this paper, we synthesized Cy derivatives, in an iterative fashion, by addition or removal of functional groups, and identified Cy deaminase resistant analogues and tested both their trypanocidal activity and their ability to resist ADA mediated deamination.

N6- modified thioaminal cordycepin prodrugs can reduce parasitemia but cannot cure the infection.

Since cordycepin gets rapidly metabolised to 3'deoxy inosine (Figure 20a) by host deaminases, we aimed in testing deaminase resistant nucleoside analogues, which have the advantage to use as standalone drugs. Of the several deaminase resistant analogues tested only N-6 thioaminal produgs of cordycepin (cordycepin 110, and116)(Figure 20b) showed antitrypanosomal activity but did not result in any curative effect in *in vivo* models (Figure 1, Paper I). It is shown that N6-substituted 9-β-D-ribofuranosylpurines (NBMPR) when phosphorylated by adenosine kinase forms toxic monophosphate (NBMPR-P) "subversive substrate"[192]. Targets of NBMPR-P are not known, but is shown to be selectively toxic against *Toxoplasma gondii*, for being substrate only to parasite adenosine kinase[192]. Since prodrugs cordycepin 110 and 116 are hydrolytically active compounds releasing parent cordycepin at various rates, they did not act as subversive substrates[193]. This might be the possible explanation for failure to cure the infection despite reduction in parasitemia.

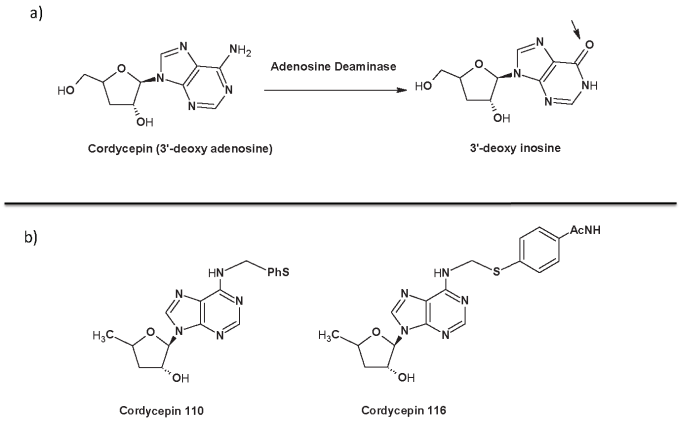


Fig 20. **a)** Conversion of cordycepin to 3'deoxy inosine by host deaminases. Deamination of N6-NH2 to hydroxyl group is indicated by an arrow. **b)** N6-thioaminal prodrugs of cordycepin 110 and 116 chemical structures[193].

The C6-NH2 and the stereochemistry of ribose in cordycepin are important for trypanocidal activity

Adenosine deaminase (ADA) catalyzes the deamination of the C^6-NH2 group of adenosine converting it into inosine (Figure 20a), hence the modification or protection of the C^6-NH2 was our initial approach for the synthesis of deaminase resistant Cy derivatives.

A C^6-NH2 modified compound (C6-N-octanoyl cordycepin, Figure 2c, Paper III) showed potent trypanocidal activity (IC_{50} = 25.9nM) but failed to cure *T.brucei* infection in mice (Figure 7, Paper III), suggesting C^6-NH2 modified compounds are prodrugs that get hydrolyzed and convert to the active cordycepin. The complete absence of C^6-NH2 in cordycepin resulted in loss of trypanocidal activity, indicating an important role of C^6-NH2 group in uptake of the drug by P2 transporter or in its intracellular salvage.

Possible modifications of the ribose sugar were studied next. The 2',3'-adenosine epoxide (Figure 2h, Paper III)showed no trypanocidal effect, thus indicating that the presence of the 2'-OH is critical to maintain activity. In addition, stereochemistry at the 2'-position was found to be critical. Inversion of the C2'-OH to yield the corresponding Cy arabinoside (Figure 2g), which ablated the trypanocidal activity of the nucleoside analogue.

The addition of fluorine at C2-position to cordycepin reduces affinity to adenosine deaminase

We next studied the possibility to modulate ADA-mediated metabolism of cordycepin through inductive modification of the adenine scaffold. The crystal structure of the mammalian ADA-adenosine complex revealed that Glu-217 and Gly-184 and the N1 and N3 of the adenine scaffold (Supplementary Figure 2, Paper III)[194] are critical for the binding and catalytic conversion of adenosine to inosine. We hypothesized that the modification of the electron density at these positions would result in decreased affinity for the enzyme. In support to our hypothesis, it has been reported that 2-fluoroadenosine and 9-b-D-a-fluoroadenine (fludarabine)[195,196] are resistant to deamination by ADA. Thus introduction of fluorine at C^2-position yielded 2-fCy (2-Fluoro Cordycepin). 2f-Cy when incubated with ADA enzyme did not result in conversion to inosine (Figure 4c, Paper III), whereas cordycepin was rapidly metabolized to inosine (Figure 4a, Paper III).

2-fCy cordycepin is deaminase resistant and is a potent trypanocidal compound

2-fCy cordycepin was deaminase resistant and showed a potent trypanocidal activity (IC_{50} = 174nM). Unlike cordycepin and the C^6-NH2 modified compounds, 2-fCy did not show increase trypanocidal activity in the presence of dCF suggesting 2-fCy was a poor substrate for ADA (Figure 3, Paper III). The trypanocidal effect 2-fCy was not solely due to addition of fluorine on the C^2-position, since 2-fluoroadenosine and 2-fluoroadenine showed no trypanocidal activity (Figure2i,j, Paper III). Adenine and adenosine have a high *K*m value for the P2 transporter[54], but the lack of toxicity of 2-fluoroadenosine and 2-fluoroadenine reinforces the importance in lacking 3'-OH group on the ribose sugar of cordycepin.

The toxicity of 2-fCy is dependent on the TbAT1 transporter and its phosphorylation by the adenosine kinase.

TbAT1$^{-/-}$ parasites showed slight shift in IC_{50} when treated with Cy. It is reported that in the absence of *TbAT1-/-*, parasites overexpress P1 like genes to balance the uptake of purines[110], this might be the reason why cordycepin is only slightly resistant to *TbAT1-/-* parasites. However resistance towards Cy was more pronounced when adenosine kinase (AK) inhibitor (ABT-702) was used in *TbAT1-/-* parasites (Figure 6a, Paper III), suggesting phosphorylation is a necessary event to elicit trypanocidal effect.

Interestingly *TbAT1*$^{-/-}$ parasites showed increased resistance to 2-fCy than Cy (Figure 6b, Paper III), suggesting that addition of fluorine at C^2-position and lack of 3'-OH might decrease the uptake by P1-related transporters. Resistance in *TbAT1*$^{-/-}$ parasites to 2-fCy was progressively increased in the presence of AK inhibitor (Figure 6b, Paper III), indicating defect in uptake and phosphorylation will effect the activity of 2-fCy. On the other hand, 2-F-adenine or 2-F adenosine were not toxic for *T. b. brucei* (Figure 2, Paper III), suggesting that the toxicity of 2-fCy is probably independent of the activity of the inosine adenosine guanosine nucleoside hydrolase (IAG-NH), involved in the alternative salvage of purines (Figure 8).

2-fCy shows good preclinical profile and can cure T.brucei infection in mice

2-fCy has very low plasma binding and has no metabolic degradation to human or mouse liver microsomes, suggesting a good therapeutic efficacy (Table 3, Paper III). 2-fCy when treated with 30mg/kg in a prophylactic *T.brucei* infection resulted in complete cure (Table 4,Paper III). Similar doses also resulted in clearance of parasites in late stage infections but relapse occurred 15 days post treatment, indicating insufficient CNS exposure of the drug with current doses. Improvements with a new dose regimen will be evaluated in the future.

With good predicted bioavailability and ability to cure mice, we present 2-fCy as a potent standalone trypanocidal molecule, which could be used as a new lead molecule in development of new therapies against HAT.

4.4 Implementation of a focused screen for the identification of new leads for the treatment of HAT (Paper IV).

Screenings of large chemical libraries provide a fast approach to develop high content lead series. Optimization of these lead series and parallel chemical synthesis allows the development of new leads with novel mechanisms of action or improved properties. As indicated above, during the past years HTS campaigns resulted in identification of lead compounds like SCYX-7158, fexinidazole, which are currently being tested in clinical trials[94,97].

We performed a focused screen of 5,500 compounds on a whole cell *T.brucei* assay that yielded several clusters of trypanotoxic primary hits. One of the clusters in the primary hits included CBK3987, which we confirmed to be a potent trypanotoxic

compound, but did not exhibit promising differential toxicity over mammalian cells. A further hit expansion on CBK3987 related molecules resulted in the identification of CBK3974 (2-Aminopyridines) and CBK201352 (2-Aminopyrazines) compounds with potent trypanocidal activity and selective window >200 fold to mammalian cells (Table I, Paper IV). The therapeutic efficacy of these two compounds was later tested in an *in vivo T.brucei* infection model. CBK3794 showed a maximum tolerated dose of 20mg/ kg once daily. Administration of this regimen reduced parasitemia levels but did not clear the infection. The related 2-aminopyrazine analogue CBK201352 showed no weight loss or adverse effects when administered 25mg/kg twice daily for 10 days and this dose was sufficient for a complete cure of the *T.brucei* infection (Figure 2A-C Paper IV).

The *in silico* and *in vitro* pharmacological studies of CBK201352 predicted that the compound has good bioavailability *in vivo*.

It has been shown that CBK201352 related classes of compounds like Piperazinylpyrazine are 5-HT_{2C} serotonin receptor agonists. 5-HT_{2C} receptors regulate mood and anxiety etc. and are widely expressed in the central nervous system[197].The effect of compounds on 5-HT_{2C} receptors in models of obesity requires CNS exposure, suggesting that the described 2-aminopyridines/2-aminopyrazines are suitable chemotypes for the development of BBB-permeable drugs.

In summary, we predict that CBK201352 has good bioavailability and is able to clear infection in mice. CBK201352 is a new class of trypanocidal that could be used as lead compound for the development of new therapies for the treatment of HAT.

4.5 Ebsulfur: a novel cytotoxic inhibitor targeting the trypanothione reductase of *Trypanosoma brucei* (Paper V).

As described in the introduction, the intracellular reducing environment of trypanosomes is maintained by a unique thiol system. In trypanosomes the trypanothione and trypanothione reductase (TryR) replace glutathione and glutathione reductase found in most other organisms[142]. TryR is an essential enzyme for the parasite and is absent in mammalian cells making it an attractive drug target. TryR is a homodimer enzyme with a large negatively charged disulfide substrate binding site[140]. However, trypanosomal death occurs only when >90% of the enzyme is depleted[142], suggesting that any inhibitor must be very potent to reach a trypanocidal level. Due to the large active site and the high intracellular levels of $T(SH)_2$, competitive inhibitors are not efficient. In this case,

irreversible inhibitors might be advantageous, since the accumulation of $T(SH)_2$ dose not control TryR inhibition.

We present novel data indicating that ebsulfur (EbS) a small Sulfur-containing molecule is a NADPH, concentration and time-dependent irreversible inhibitor of the *T. brucei* TryR.

EbS is toxic to T.brucei but not to other trypanosomatids

By measuring luciferase activity in recombinant *T.b. brucei*, we found that EbS caused a rapid trypanocidal effect, reaching submicromolar IC_{50} levels within 4-6 hrs of incubation (Figure 1A, Paper V). We next measured IC_{50} of Ebselen (EbSe) and EbS on *T.b. brucei* and other trypanosomatids (*T. cruzi & Leishmania*) using WST-1 reagent. We observed that EbS showed more than 100 fold higher inhibition of *T. b. brucei* growth than EbSe (Table I, Paper V). EbS was selectively toxic to *T. b. brucei* and other human pathogenic sub species but not to *T.cruzi & Leishmania*. To understand such selective trypanocidal effect of EbS, we later measured the trypanothione levels in *T. b. brucei & T. cruzi*. We observed that $T(SH)_2$ contents in blood stream *T.brucei* were 10 times lower compared with those in *T. cruzi* (Table II, Paper V), indicating that *T.brucei* has a weak thiol metabolism system, making it more susceptible to the thiol targeting drugs.

EbS induces programmed cell death by accumulation of ROS

EbS induces programmed cell death (PCD) in trypanosomes (Figure 1B-C, Paper V). It has been reported that in mammalian cells as well as in trypanosomes ROS can initiate a signal transduction pathway leading to PCD[198–200]. We measured accumulation of ROS in parasites treated with EbS (Figure 3A, Paper V). Since the trypanothione cascade protects parasites against oxidative stress, we hypothesized that the accumulation of ROS is a consequence of deregulation of the trypanothione system. EbS treated parasites showed low levels of reduced non-protein thiol and also showed reduced TryR activity (Figure 1D-E, Paper V), indicating EbS targets trypanothione pathway. We also observed complex of EbS with TryR using mass spectrometry confirming that EbS targets TryR (Figure 2G & Supl Fig S2 , Paper V).

EbS is an irreversible inhibitor of trypanothione reductase

To understand the interaction between TryR with EbS, we studied the effects of EbS on a pure TryR enzyme. We found that EbS inhibited the activity of recombinant TryR in a time and concentration-dependent manner (Figure 2A, Paper V). The activity of inactivated TryR was not recovered after removal of EbS by filtration through Sephadex-G25, even in presence of 1mM DTT, indicating that EbS is an irreversible inhibitor of the enzyme. Since TryR reduces $T(SH)_2$ to regulate the parasite thiol metabolism, we further examined if $T(SH)_2$ regulates the inhibition of TryR by EbS. Addition of $T(SH)_2$ protected TryR in concentration-dependent manner (Figure 2D, Paper V). Thus, even if EbS is an irreversible inhibitor of TryR, the inhibition of TryR by EbS is regulated by pre-existing substrate levels. But, due to the low amounts of $T(SH)_2$ in *T.brucei* probably contribute to the susceptibility to EbS, since *T. cruzi* that contained high $T(SH)_2$ and TryR levels was relatively resistant to this molecule (Table II, Paper V).

EbS converts TryR an antioxidative disulfide reductase into a prooxidative enzyme

It has been previously reported that in the absence of trypanothione, TryR show enhanced NADPH oxidase activity when incubated with naphthoquinones[178]. Similarly, a NADPH oxidase activity of TryR was observed when incubated with EbS. Compounds which have NADPH oxidase activity when interact with TryR are called "subversive substrates"[155,178]. Subversive substrates when reduced produce free radicals and react with molecular oxygen to produce superoxide anion radicals. We speculate that with NADPH oxidase activity and subsequent inactivation of the enzyme, EbS converts TryR an antioxidative disulfide reductase into a prooxidative enzyme (Figure 4A, Paper V). Supporting this information we also observed oxidised TryR complex with EbS using mass spectrometry (Figure 2G, Paper V). Thus we observed increased production of H_20_2 with EbS but diminished in the presence of both EbS and $T(SH)_2$ (Figure 4A, Paper V) suggesting that $T(SH)_2$ protects from the NADPH oxidase activity of EbS.

An intrinsic peroxidase activity of EbS was also evidenced when TryR, and $T(SH)_2$ were incubated (Figure 4B, Paper V). High EbS levels inhibited such activity. To understand this inhibition, each component of the assay was freshly added to the existing reacting mixture. The inactivation of TryR was confirmed by the addition of fresh TryR that recovered the peroxidase activity while addition of $T(SH)_2$ did not (Figure 4C, Paper

V). Thus, the inhibition of peroxidase activity was confirmed due to inactivation of TryR by EbS (Figure 4C, Paper V).

Primary detoxification of ROS in parasites is mediated through tryparedoxin (TXN) and tryparedoxin peroxidases (TXNPx)[147,148]. Activation of these enzymes is through the cascade of trypanothione pathway involving TryR. Inactivation of TryR was more pronounced in the presence of external H_20_2, indicating accumulation of ROS due to impairment of classical peroxidase activity might have a synergistic effect in inhibiting TryR (Figure 4D, Paper V). Thus, inhibition of TryR activity might contribute to the increased levels of intracellular ROS levels leading to PCD.

The combination of EbS 24 and nifurtimox clears T.brucei infection

We next tested the efficacy of EbS in *in vivo* infection models. Treatment with EbS decreased the levels of parasites but did not clear parasitemia (Figure 5A,Paper V). EbS is poorly soluble and might have an insufficient exposure *in vivo*. Due to poor solubility of EbS we synthesized different EbS analogues. One of them, EbS24 was soluble and retained the trypanotoxic activity of EbS in vitro. Administration of EbS24 alone could not clear parasitemia but when administered together with nifurtimox resulted in a complete cure of infection in a fraction of treated mice, while none of those treated with nifurtimox alone was cured (or showed reduced parasitemia levels). Both compounds have a synergistic activity in vitro (Figure 5C, Paper V). However, when parasites were treated with nifurtimox no increased ROS levels were detected, suggesting both drugs have independent mechanism of killing (Suppl Fig S3, Paper V).

It has been reported that the EbS analogues have a good oral bioavailability[201] suggesting EbS and its derivatives could be new leads in development of drugs targeting oxidative defense of parasites.

5. CONCLUDING REMARKS

Human African trypanosomiasis is fatal if untreated. In the past few years, great advances have been made to control HAT, with strict implementation of the treatment regimens and vector control. However, it is estimated that approximately 70 million people are at risk for contracting HAT in the regions of sub Saharan Africa[6]. The presence of a chronic or asymptomatic forms of disease with low and fluctuating parasitemia producing apparently aparasitemic serological individuals might also pose a problem in disease control since these will remain untreated due to the unacceptable toxicity of current drugs and the difficult treatment regimens.

With only two drugs available for the late stage, there is an urgent need for the development of new safe, cost effective and non-toxic drugs.

The studies in this thesis identify diverse lead compounds that are effective in curing murine models or in selectively killing *T.brucei* that could be used for further development of drugs against HAT.

The inability of trypanosomes to synthesize purines *de novo* was exploited as a therapeutic target. We showed that treatment with cordycepin when protected from deamination by the adenosine deaminase by deoxycoformycin cures late stage murine models of African Trypanosomiasis. This nucleoside combination is efficient in curing both human pathogenic subspecies and can be administered orally. Besides the results from preclinical experiments described in our manuscript, cordycepin is cheap and deoxycoformycin is an FDA approved drug. The dose of deoxycoformycin required for treatment was far below the maximum tolerated dose. Thus, this doublet can provide the foundation for an alternative treatment for the encephalitic stage of HAT.

We aimed to generate novel cordycepin analogs, which retain trypanotoxic activity while gaining resistance to ADA-mediated degradation. The trypanocidal activity of cordycepin is exquisitely dependent on a number of critical recognition motifs to elicit trypanocidal effects (e.g. ADA, *TbAT1*, AK etc.) *in vivo* without causing general cytotoxicity. Most compounds evaluated were either inactive or caused ADA inhibition. 2-Fluoro cordycepin (2-fCy) exhibited a potent trypanocidal activity in vitro, was dependent on adenosine kinase activity on the uptake by the *TbAT1* receptor and was resistant to ADA degradation, as it interfered with ADA binding. 2-fCy showed good *in vitro* preclinical profile and was also effective on human pathogenic subspecies.

Administration of 30 mg/kg 2-fCy but not cordycepin cured an acute *T brucei* infection in mice, indicating 2-fCy can be used as standalone drug. Our data strongly suggest that cordycepin-analogues should be further tested for HAT treatment.

Screening drug libraries using whole cell assays might provide new classes of anti parasitic drugs. We performed a focused screen of a drug library and found several 2-aminopyrazines/2-aminopyridines as promising leads. Specifically CBK201352 was trypanotoxic for the human pathogenic subspecies. CBK201352 has good predicted preclinical profile and is effective in curing an acute *T.brucei* infection in mice. CBK201352 and related compounds could be new leads for further developing drugs to treat HAT.

In trypanosomes, the trypanothione systems replace the glutathione and thioredoxin systems found in most other organisms in the defense against oxidative stress. Thus the trypanothione pathway contributes to be an attractive pharmacological target. We showed that Ebsulfur (EbS) a sulfur-containing small molecule is an irreversible inhibitor of trypanothione reductase (TryR). EbS also stimulated the NADPH oxidase activity of TryR. EbS treatment resulted in the accumulation of ROS in parasites. EbS was toxic for the human pathogenic subspecies of *T.brucei* but not for mammalian cells and cured *T.brucei* infection if co administered with nifurtimox. Thus, EbS and its derivatives could be new leads for development of drugs targeting oxidative defense in parasites.

Thus the drugs investigated in this thesis work are active against both human pathogenic subspecies, have good predicted bioavailability and in some cases can cure murine late stage encephalitic African Trypanosomiasis. Further development of these drugs could result in innovative therapeutic treatment for Human African Trypanosomiasis.

6. ACKNOWLEDGEMENT

I would like to express my sincere gratitude to each and every one who directly or indirectly encouraged me to finish my thesis at MTC.

First of all I would like to thank my supervisor **Prof. Martin Rottenberg** for transforming me from a master student to a PhD student. Thank you for accepting me as your student and listening to my wild ideas. You have been a great teacher all these years and I have learnt a lot of things. I enjoyed each and every minute sharing the office space, listening to your music, and playing tennis in the mornings and all those wonderful skiing trips you organized.

I would like to thank my co supervisors **Prof. Krister Kristensson**, **Dr. Sonia Lain** and **Prof. Björn Andersson** for enthusiastic encouragement and useful suggestions for my research work.

I would also like to thank:

Berit Olsson Aka **"Big Berit"** you are a wonderful person one can ever have in the lab. You have been like a mother all these years and with your charming presence I have never missed my family back in India. Thank you for teaching me Swedish and your suggestions for cooking.

Berit Carow Aka **"Small Berit"** for the sister support during these years. You have lot of patience in listening to my Indian stories and adventures during lunch and coffee breaks. If you are coming to visit India with **Ann Kathrin**, I will show you the real world.

Frank, for teaching immunology and helping out with various things in the lab. I will remember all my life "its Suman's fault" ☺.

Carina my Partner in crime, it was so much fun to work with you. Thank you for all your moral support and teaching me how to dance.

Lars, Thomas, Esther, Birger, Anna Lena, Arnika, Arne, Lu, Thomas, it was fun working in collaboration with you. Thank you so much for your technical help and your input for my research work.

Hilda, for teaching about the project and the fun time in the lab.

Marcela, for helping with all the cloning work and sequencing.

Pouria & Gao my successors for the new Spanish swearing ☺.

Tony, Susanne, Vishnu, Katja, for stimulating discussions in the journal clubs.

My students **Clemens, Rebecca, Liban, Fathima**, and previous lab members **Ahmed, Daniel, Christian, Ann Kathrin, Katrin, Sukumar, Tang Bin, Yeh, Cindy, Frieda, Steffi, Eni.**

My best friends **Kanth & Deepa,** thank you so much for believing in me and constantly encouraging to do all the wild things.

Our previous lab corridor members, **Speranta, Sem, Naeem, Syed, Aurel, Mikael Rhen,** for all the good times.

My Indian gang, **Anuj, Ranjana, Shastry, Pavan, Basi babai, Madhavi, Harsha, Pradeep, Jagadeesh, Suhas, Ashwini, Lalit, Dipti, Shahul, Sunitha, Shakthi, Vivek, Saranya, Rajender, Nalini, Ajay, Him, Sridhar, Aditya** and **Chaniya ☺,** thank you so much for all the fun activities and delicious Indian food.

My Pub crew, **Mario, Miriam, Chaniya, Katja, & Anuj**, guys we redefined entertainment at MTC.

People at MTC, **Benedict, Jess, Thomas, Mark, Hannes, Koyoko, Anna, Inge, Cath, Marijke, Arnika, Sadia, Siti Mariam, Birgitta, Patrick, Jolanta, Adil, Lech, Katrin.**

Thanks to the people at animal unit, **Anna Karin, Magan, Kent, Torun**

Nomei, Jorma and Orlando for all the fun with frisby golf and best parties.

My corridor mates **Faiz, Lina, Emma, Victor, Athena, kaviresh, Hoda, Teresa, Simon, Farooq, Adam**, for all the fun in the corridor. **Faiz**, thank you for all the pranks, bouldering sessions and pizza eat outs. Lina & Victor you guys are the best partners to do all the crazy stuff.

My friends at KTH, **Yashas, Hongqian, Zhang Xu, Anna,** and **Sumire.**

My Brother **Ajay**, thank you so much for supporting for my Masters program at KTH. You are true inspiration for me in many things. My sister in law **Swetha** & nephew **Ishaan**, you guys brought new color into our family. Finally I would like to thank my parents (**Sri Hari & Padmaja**) who were there for me all the time and giving me freedom to follow my own choices.

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