NEGLECTED TROPICAL DISEASE CHEMOTHERAPY: MECHANISTIC CHARACTERIZATION OF ANTITRYPANOSOMAL DIHYDROQUINOLINES AND DEVELOPMENT OF A HIGH THROUGHPUT ANTILEISHMANIAL SCREENING ASSAY

DISSERTATION

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By

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ABSTRACT

Human African trypanosomiasis (HAT) and leishmaniasis are identified by the World Health Organization (WHO) as neglected tropical diseases (NTDs), together with Chagas disease and Buruli ulcer. These NTDs mostly affect people in remote or rural area, and there are very limited control and therapeutic options. The investment on research and development against NTDs is insufficient.

Human African trypanosomiasis (HAT) is a vector-borne parasitic disease caused by *Trypanosoma brucei* subspecies. Transmitted by the tsetse fly, the disease mainly affects rural populations in sub-Saharan Africa and is fatal if untreated. New drugs are needed against HAT that are safe, affordable, easy to administer, active against first and second stage disease, and effective against both subspecies of *T. brucei* (11, 139).

From medicinal chemistry investigation in Karl Werbovetz group, several N1-substituted 1,2-dihydroquinoline-6-ols were discovered displaying nanomolar IC_{50} values *in vitro* against *T. b. rhodesiense* and selectivity indexes (SI) up to >18,000 (39). OSU-40 (1-benzyl-1,2-dihydro-2,2,4–trimethylquinolin-6-yl acetate) is selectively potent against *T. b. rhodesiense in vitro* (IC_{50} = 14 nM, selectivity index = 1700), and has been proposed to cause the formation of reactive oxygen species (ROS) in African trypanosomes. In the

present study, we sought to provide further support for the hypothesis that OSU-40 kills trypanosomes through oxidative stress. Inducible RNAi interference (RNAi) was applied to down-regulate key enzymes in parasite antioxidant defense, including trypanothione synthetase (TbTryS) and a superoxide dismutase (TbSODB). Both TbTryS RNAi-induced and TbSODB RNAi-induced cells showed impaired growth and increased sensitivity towards OSU-40 by 2.4-fold and 3.4-fold respectively. Decreased expression of key parasite antioxidant enzymes was thus associated with an increased sensitivity to OSU-40, consistent with the hypothesis that OSU-40 acts through oxidative stress. Finally, the dose-dependent formation of free radicals was observed after incubation of *T. brucei* with OSU-40 utilizing electron spin resonance (ESR) spectroscopy. These data support the notion that the mode of antitrypanosomal action for this class of compounds is to induce oxidative stress.

The ultimate goal of our work with antitrypanosomal dihydroquinolines is to develop a new drug against HAT. A series of studies were carried out with representative dihydroquinolines to evaluate their drug-like properties to aid the development of these compounds as prospective new HAT treatments. The kinetic solubility assay confirmed that the salt forms (OSU-36•HCl and OSU-95•HCl) showed improved solubility compared to the esters (OSU-40 and OSU-75), respectively. The liver cytotoxicity assay showed that dihydroquinolines were highly selective on trypanosomes over human liver cells, suggesting the compounds were not particularly more toxic to liver cells. Clonal OSU-40 resistant *T. brucei* parasites were developed through 22-month selection under

increasing OSU-40 pressure. These clonal cells showed dramatically lowered sensitivity to OSU-40 and OSU-36•HCl and, to our surprise, higher sensitivity to both nifurtimox and H_2O_2. Whole genome sequencing will be performed to determine differences between the parental line and resistant line.

Leishmaniasis is a parasitic disease caused by more than 20 species of the protozoan *Leishmania*. Canine leishmaniaisis caused by *Leishmania infantum* is one of the major zoonotic diseases that lead to death in dogs. Drugs applied in therapy for canine leishmaniasis are not satisfactory. Studies on new combination therapy (77) and canine vaccines (25, 80, 104) have been reported targeting canine leishmaniasis, but discovery and development of novel drugs against this disease caused by *Leishmania infantum* has not been reported.

High throughput screening assays on *L. infantum* and *L. donovani* were designed, developed and validated in a rapid and simple 384-well format with a satisfactory robustness (Z' = 0.71 and 0.86, respectively). From the screening of a 427-member library, six compounds (2, 14, 17, 19, 20 and 21) exhibited IC_{50} values that are comparable to pentamidine (lower than 5 μM) against *L. infantum* and/or *L. donovani.* Comparison of *L. infantum* screen to *L. donovani* screen indicates that screen against *L. donovani* is not an ideal approach to identify hits on *L. infantum* because of a high false positive rate. The antileishmanial screening assay against *L. infantum* developed in this study is a good initiation point leading to new drug candidates to the discovery pipeline for canine leishmaniasis.

THIS DOCUMENT IS DEDICATED TO

MY PARENTS, QIN GAO AND NINGSHENG HE

AND MY HUSBAND, SU-LIN LEE

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FIELDS OF STUDY

Major Field: Pharmacy

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: HUMAN AFRICAN TRYPANOSOMIASIS AND CHEMOTHERAPY

Human African trypanosomiasis (HAT) is a vector-borne parasitic disease caused by *Trypanosoma brucei* subspecies, *T. b. rhodesiense* and *T. b. gambiense*. The trypanosome proliferates in the host's hemolymphatic system during the first stage of HAT and invades the central nervous system in the second stage. Transmitted by the tsetse fly, the disease mainly affects rural populations in sub-Saharan Africa and is fatal if untreated. Although according to the World Health Organization the number of new cases has dropped to just over 7000 during 2010 (135), very few drugs are available to treat HAT (8, 11, 139). Pentamidine and suramin, both developed in the early 20th century, are used against first stage disease, while melarsoprol and eflornithine are used to treat second stage disease. These drugs are far from satisfactory due to limitations such as severe toxicity, poor efficacy, acquired resistance, rising failure rates, and lack of availability (8, 11, 139). With high efficacy and a good safety profile, nifurtimox-eflornithine combination therapy (NECT) was introduced as first-line treatment for second stage HAT caused by *T. b. gambiense* in 2009 (11, 84, 139). However, administration of NECT is relatively complicated and its efficacy towards *T. b. rhodesiense* is still questionable (11, 139). Thus, new drugs are needed against HAT that are safe, affordable, easy to

administer, active against first and second stage disease, and effective against both subspecies of *T. brucei* (11, 139).

In this chapter, a brief introduction of the disease is given, including parasite transmission and life cycle, clinical manifestations, epidemiology, diagnosis and control of HAT. The current chemotherapy and drug discovery efforts are reviewed in detail. Finally, the discovery of dihydroquinolines provides the background for further studies on the mode of action and drug-like properties of this series of compounds in the following chapters.

1.1 HUMAN AFRICAN TRYPANOSOMIASIS

1.1.1 Parasite transmission and life cycle

As shown in Figure 1.1 (14), a tsetse fly infected with *Trypanosoma brucei* injects metacyclic trypomastigotes into host tissue when taking a blood meal. The parasites then pass from the lymphatic system into the bloodstream, where the metacyclic trypomastigotes transform into bloodstream form *T. brucei*. Bloodstream form trypomastigotes proliferate rapidly by binary fission in the blood while being carried throughout the body. When taken up by a tsetse fly during a blood meal on the infected host, the parasites pre-adapted for life in the insect vector differentiate into procyclic form trypanosomes. The parasites then replicate by binary fission in the fly's midgut and transform into epimastigotes after they leave the midgut. When epimastigotes migrate to

the salivary glands, they may be introduced into the host as the fly takes a blood meal, completing the life cycle of *T. brucei.* Humans are the only mammalian host of *T. b. gambiense*, while *T. b. rhodesiense* infection is zoonotic with a variety of domestic and wild animal reservoirs. On the other hand, *T. b. brucei* infects livestock, not humans.

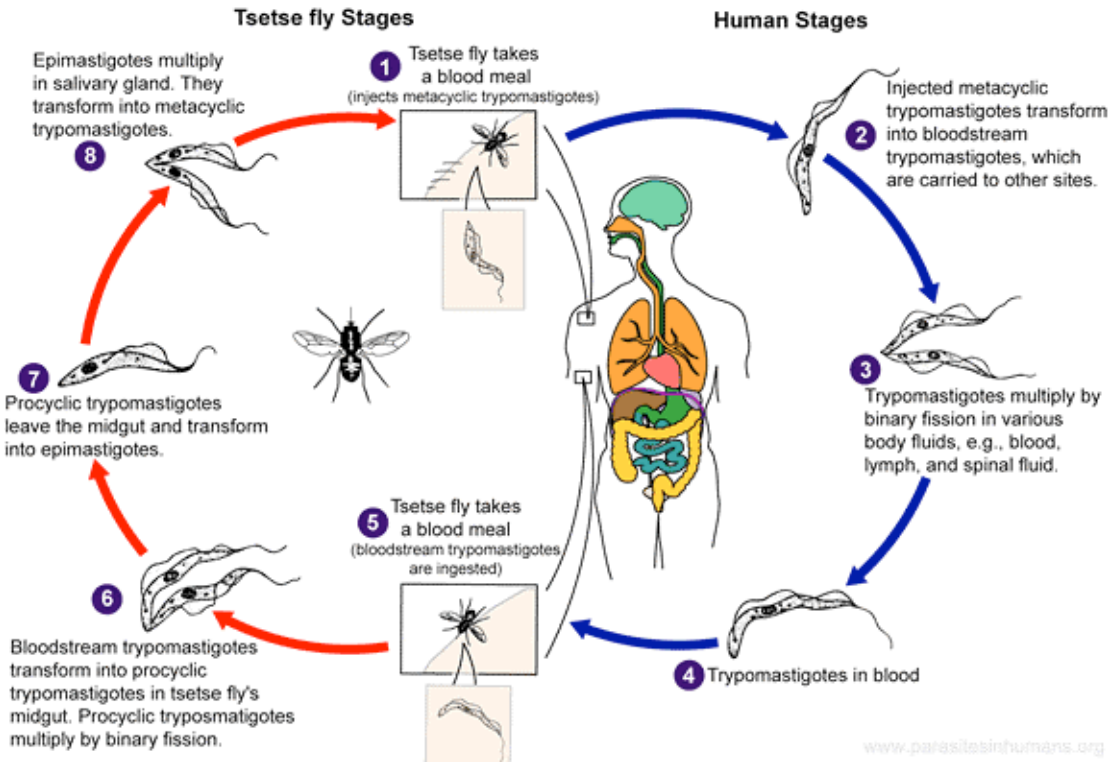

Figure 1.1 Life cycle of *Trypanosoma brucei.*

[Source: DPDx website, Division of Parasitic Diseases and Malaria (DPDM), Centers for Disease Control and Prevention (CDC) (14)]

1.1.2 Clinical manifestations

Human African trypanosomiasis has two clinical stages. In the first stage, the parasites are only found in the bloodstream of the host and have not yet entered the central nervous system. The disease progresses to the second stage when the parasites cross the blood-brain barrier and infect the central nervous system.

Although the two subspecies of *T. brucei* that cause disease in humans, *T. b. rhodesiense* and *T. b. gambiense*, are indistinguishable in their morphology and transmission, the diseases they cause in humans progress at different rates and have distinct clinical features (14, 17, 100). *T. b. rhodesiense* causes infection in East and South Africa, and the infection progresses rapidly. Some patients develop a large sore at the site of the tsetse fly bite. Fever, headache, loss of appetite, muscle and joint aches, and enlarged lymph nodes commonly develop within 1-2 weeks of the infective bite. HAT caused by *T. b. rhodesiense* progresses to the second stage within a few weeks of infection, when patients suffer from mental deterioration, disruption of sleeping cycles and other neurologic problems. Death can take place within months. *T. b. gambiense* causes infection in West and Central Africa and the disease is characterized by chronic progression. Mild symptoms, such as intermittent fever, headaches, and malaise occur at first; central nervous system involvement usually requires 1-2 years. Neurologic signs include progressive confusion, sleep abnormalities, difficulty in maintaining balance or

walking, and hormonal imbalances. *T. b. gambiense* HAT leads to death in approximately 3 years and the untreated infection in some cases can last longer than 6 years.

1.1.3 Epidemiology

Restricted by the presence of the vector tsetse fly, HAT distributes in mostly poor and remote rural areas in Africa. For reasons that are not well understood, there are places where the tsetse fly exists but HAT is not found. The epidemiological characteristics are different between the two subspecies of *T. brucei* parasites. *T. b. rhodesiense* HAT occurs in 13 Eastern and Southern African countries (Figure 1.2), including Uganda, Rwanda, Burundi, the United Republic of Tanzania, Zambia and Malawi (71). *T. b. gambiense* leads to chronic infection in countries of Western and Central Africa (Figure 1.2), specifically the Democratic Republic of the Congo, the Republic of Congo, Angola, the Central African Republic, and the Republic of South Sudan (17).

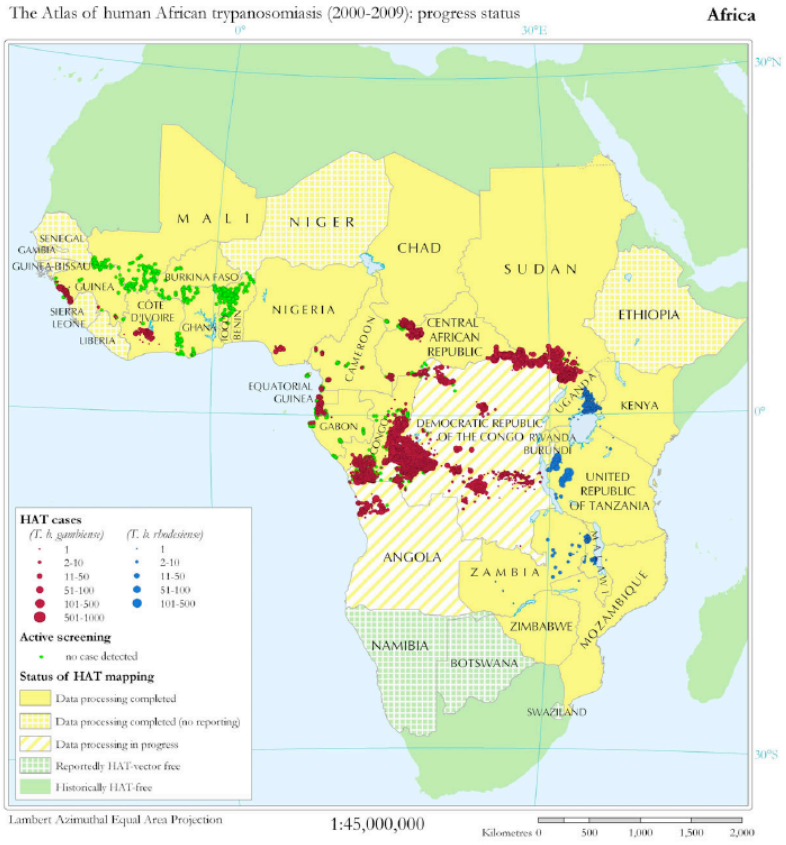

Figure 1.2 Distribution of HAT caused by *T. b. rhodesiense* and *T. b. gambiense.*

[Credit: WHO/Food and Agriculture Organizations of the United Nations; Reprint with permission from BioMed Central Ltd: International Journal of Health Geographics (110), copyright (2010)]

In the early 19th century, epidemics of HAT occurred in Africa from 1896-1906 and in the 1920s (Figure 1.3). The disease was almost eliminated in the mid-1960s because of systematic screening, treatment and follow-up throughout the continent. However,

disease surveillance relaxed after the reduction of the number of HAT cases, leading to a flare-up of the disease over the following years until the late 1990s (Figure 1.3) (112). According to the World Health Organization (WHO), almost 40,000 cases were reported in 1998, and 300,000 cases were estimated to be undiagnosed and untreated in that year (132). In South Sudan, Angola and the Democratic Republic of the Congo, prevalence reached 50% in certain areas. HAT caused the most or second to the most deaths in these villages, in some areas more than the number of deaths caused by HIV infection (132). Surveillance, control, diagnosis and treatment of HAT were then strongly reinforced with the efforts of WHO, national control programs, bilateral cooperation and the public and private sector. This led to a reversal of the rising trend of disease incidence from the late 1990s to the beginning of the 21st century (111, 112). The number of HAT cases fell below 18,000 in 2004 and with this continuing trend, the number of cases dropped below 10,000 in 2009 for the first time in 50 years (132). During 2010, 7139 new cases of HAT were reported to WHO (135).

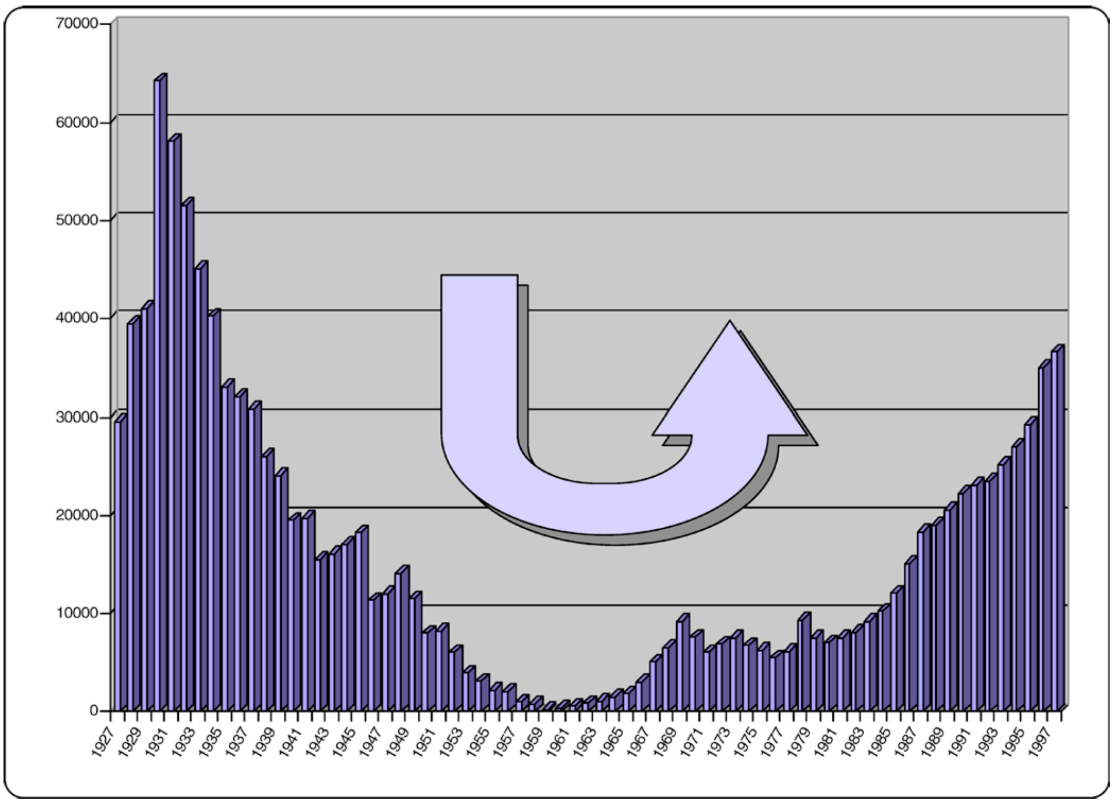

Figure 1.3 New cases of HAT reported between 1927 to 1997.

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1.1.4 Diagnosis and control

1.1.4.1 Diagnosis

Lacking specific clinical features, laboratory tests are required to diagnose HAT infection. HAT diagnosis relies on the observation of trypanosomes in body fluids or

tissues under the microscope. Diagnosis of infection with *T. b. rhodesiense* and *T. b. gambiense* share the same general principles, and they both require three steps: screening, parasitological confirmation and stage determination (17, 71).

T. b. rhodesiense infection is easier to detect due to the higher parasite load that occurs. Screening is carried out to identify suspected cases based on non-specific symptoms and a history of exposure. Parasitological confirmation rests on microscopic detection of parasites because of the high density of parasites in the blood for *T. b. rhodesiense* infection. Serologic testing is not applied in this case (14).

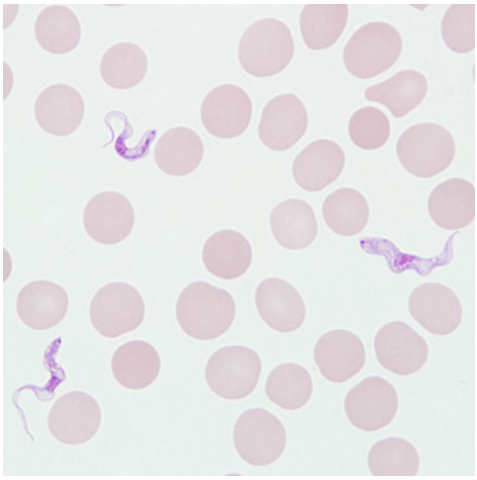


Figure 1.4 *Trypanosoma brucei* ssp. in a thin blood smear stained with Giemsa.

[Source: DPDx website, Division of Parasitic Diseases and Malaria (DPDM), Centers for Disease Control and Prevention (CDC) (14)]

Following the same general steps, diagnosis of *T. b. gambiense* HAT differs from the above methods used for *T. b. rhodesiense* infection in the following points (14, 17, 71). First, screening in endemic areas of Africa is carried out with a rapid serological test, the card agglutination test. Due to the limited specificity of this test, all individuals with positive results in the card agglutination test still require parasitological confirmation. Microscopic examination of cervical lymph node fluid or blood is carried out to search for trypanosomes. Blood concentration techniques are often needed due to the low parasite load in *T. b. rhodesiense* infection.

All individuals diagnosed with HAT are required to undergo cerebrospinal fluid examination to determine whether the central nervous system is involved for stage determination of the disease (17, 71). Different treatments will be administered depending on the disease stage. Overall, the diagnostic approaches currently in use are complex for rural sub-Saharan Africa and more practical tests are urgently needed for accurate HAT diagnosis.

1.1.4.2 Control and surveillance

The strategies for control and surveillance of HAT include case screening, diagnosis, treatment, case follow-up, vector control, and disease reservoir control (14, 133). These

strategies are utilized at different levels, depending on the epidemiological status, the subspecies causing the infection, and local environment, such as geographic, political and climatic factors. *T. b. rhodesiense* infection is zoonotic with a variety of domestic and wild animal reservoirs, leading to difficulty in reservoir control. With humans as the main disease reservoir, *T. b. gambiense* HAT is mainly controlled by active population screening and treatment of diagnosed cases.

1.2 CHEMOTHERAPY

Without chemotherapy, HAT caused by both *T. b. rhodesiense* and *T. b. gambiense* most often leads to death. In the absence of a vaccine, the only options to control and treat HAT relied on four drugs in the 20th century, including pentamidine and suramin for first stage HAT, and melarsoprol and eflornithine for second stage disease (8, 11, 55). In 2009, nifurtimox-eflornithine combination therapy (NECT) was introduced as first-line treatment for second stage *T. b. gambiense* HAT. The chemical structures of these drugs are shown in Figure 1.5.

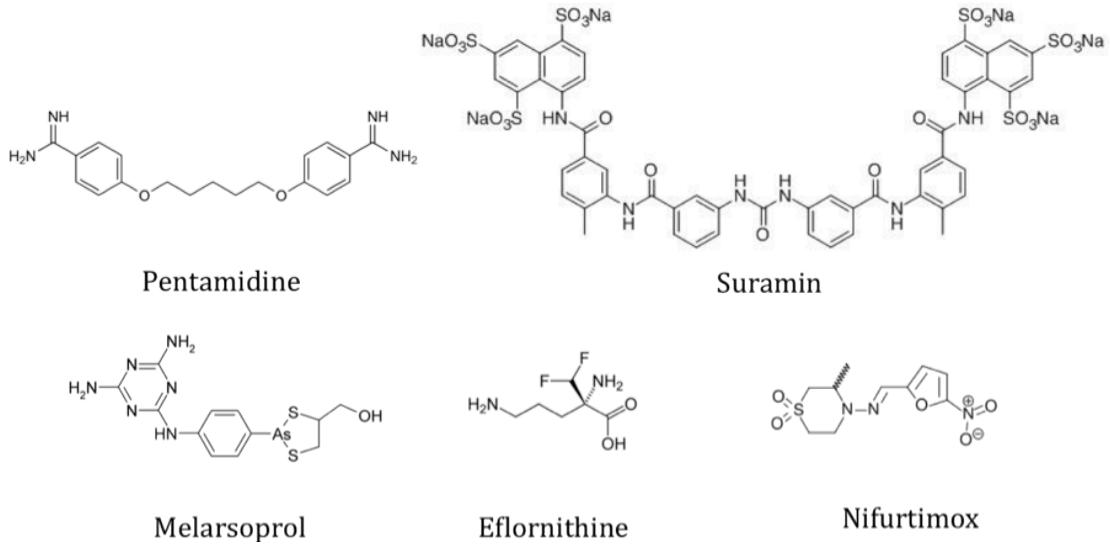


Figure 1.5 Chemical structures of the drugs used in HAT chemotherapy.

1.2.1 Chemotherapy for first stage HAT

1.2.1.1 Pentamidine

Pentamidine isethionate was introduced in the 1940s. It is a symmetric diamidine derivative. The uptake of this drug is carrier mediated primarily via the P2 aminopurine permease, and also a high-affinity pentamidine transporter 1 and a low-affinity pentamidine transporter 1 (26). This explains the observation that high concentrations of pentamidine appear in trypanosomes when the parasites were incubated in low concentrations of the drug. The accumulation of pentamidine in trypanosomes may be the primary reason for its selectivity over host cells. Although the mechanism by which

pentamidine concentrates in the parasite has been studied in detail, the mode of action of this compound is still not fully understood (131). Multiple targets could contribute to pentamidine's trypanocidal effects. The activity of this drug was suggested to be related to effects on the parasite mitochondrion, such as interacting with mitochondrial kinetoplast DNA (68, 109).

Pentamidine has a broad range of antimicrobial activity. It is also used for the treatment of leishmaniasis and *Pneumocystis carinii* pneumonia in AIDS (21). For the treatment of HAT, pentamidine is only used to treat first stage *T. b. gambiense* infection because it showed relatively low activity on *T. b. rhodesiense* (55) and because the concentration of pentamidine in the central nervous system was not high enough to effectively kill the parasites during the second stage HAT (103). Compared to melarsoprol for late stage HAT, pentamidine is well tolerated. However, it still shows significant toxicity in patients when given by intramuscular injection, causing adverse reactions such as abdominal pain, diarrhea, nausea etc. Life threatening hypoglycemia is also one of the frequently reported adverse reactions (8).

1.2.1.2 Suramin

Suramin, the oldest drug in HAT chemotherapy, was introduced in the 1920s. It is a polysulphonated symmetrical naphthalene derivative. The uptake of suramin most likely relies on receptor-mediated endocytosis, leading to concentration of the drug in the

parasites (34). This may be the primary reason for its selectivity on trypanosomes over host cells. The mode of action of suramin is not well understood. The absence of field resistance suggests a multiple-targeted mode of action. With six negative charges, suramin may be able to interact with many enzymes via electrostatic interaction. On the other hand, the drug is significantly less active against procyclic form *T. brucei* (107), which differs from bloodstream form parasites in that glycolysis is not essential for survival (9). Thus, glycolysis is also a proposed target.

Suramin is used for the treatment of first stage *T. b. rhodesiense* HAT. Besides this clinical application, it is reported to be active in other conditions, such as androgen-independent prostate cancer (63). Suramin is not effective against late stage HAT because the drug does not cross the blood brain barrier. The adverse reactions caused by suramin include nausea, vomiting, rash, hyperglycemia, and neurological complications such as peripheral neuropathy. The US Food and Drug Administration disapproves the use of suramin in prostate cancer because of adverse reactions (63). However, with a short regimen the drug is tolerable for HAT treatment.

1.2.2 Chemotherapy for second stage HAT

1.2.2.1 Melarsoprol

Melarsoprol was introduced for HAT chemotherapy in 1949. It is a melaminophenyl

based trivalent arsenical. Melarsoprol is a prodrug when administered in vivo, being rapidly converted to melarsen oxide. Melarsen oxide enters the parasites primarily via the P2 aminopurine transporter with possible secondary routes reported in case of loss of the P2 transporter (12, 26, 74). The drug results in a rapid lytic effect on trypanosomes, but the mechanism of killing is not well understood. It is reported to interact with trypanothione, a small molecule that plays essential roles in antioxidant systems in trypanosomatids but not in host cells (36). However, it is not known whether the interaction with trypanothione is the primary reason for its toxicity to trypanosomes.

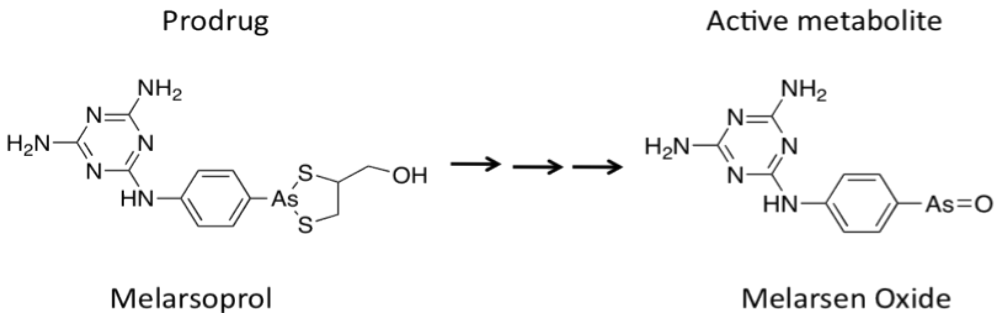


Figure 1.6 Chemical structures of melarsoprol and melarsen oxide.

Melarsoprol is the only drug that is active against second stage HAT caused by both *T. b. rhodesiense* and *T. b. gambiense*. Because of serious toxicity, the drug is reserved only for the treatment of late stage *T. b. rhodesiense* infection, and it is recommendedagainst

T. b. gambiense when eflornithine is unavailable. Side effects such as fever, vomiting, abdomimal cramping and malaise can appear rapidly after administration. More severe toxicity effects occur due to reactive encephalopathy, which results in convulsions and other neurological conditions leading to coma and death. This serious adverse effect occurs in 10% of the patients receiving the treatment and can lead to death in 5% of the total number of treated patients (91). Due to these life threatening side effects, hospitalization is required during melarsoprol treatment and patients are subjected to follow-up for 2 years after treatment.

1.2.2.2 Eflornithine

Eflornithine, or D, L-α-difluoromethylornithine, introduced in 1981, is the only new drug that has been registered for the treatment of HAT in the past 50 years. It is a fluoro-substituted analogue of the amino acid ornithine. Compared to pentamidine, suramin and melarsoprol, eflornithine is the only drug in HAT chemotherapy with an established mode of action. Eflornithine kills African trypanosomes through inhibition of ornithine decarboxylase (ODC), an essential enzyme in the polyamine biosynthesis pathway (5, 6) (Figure 1.7). Polyamines are essential for eukaryotic cell growth. Although eflornithine inhibits both the mammalian and parasite ODCs, it selectively kills the parasites because the turnover of ODC is slower in *T. b. gambiense* than in mammalian host cells (43). In addition, polyamines play a unique role in the trypanosomatids in that spermidine reacts

with glutathione to form trypanothione (35). Polyamine and trypanothione biosynthesis pathways will be discussed in detail in section 1.3.3 as potential therapeutic targets.

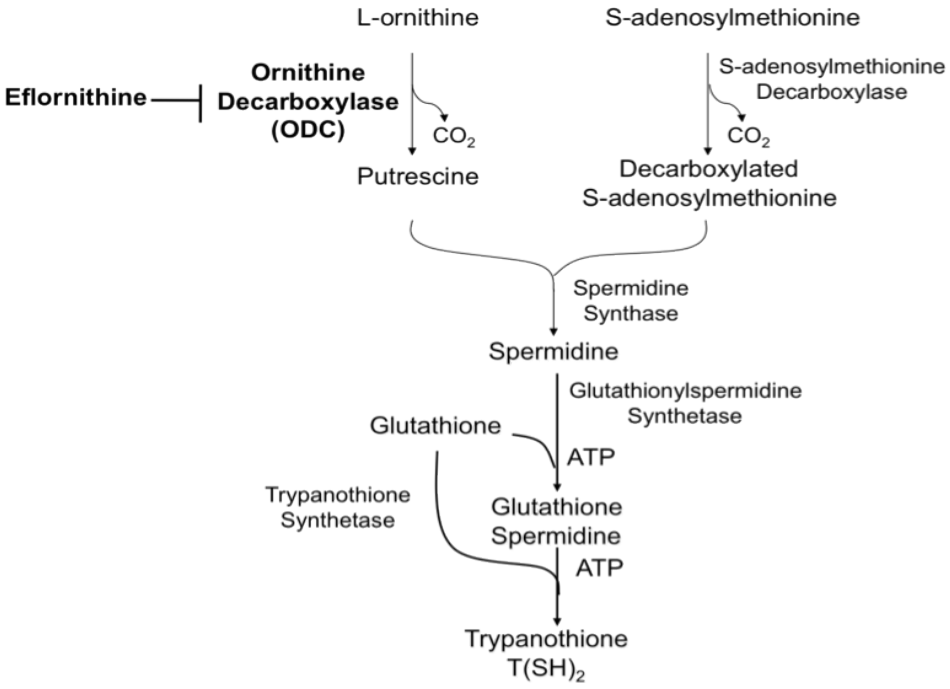


Figure 1.7 Mode of action of eflornithine shown in trypanosomal polyamine biosynthesis pathway.

Eflornithine is used to treat late stage *T. b. gambiense* HAT, because it is not active against *T. b. rhodesiense* infection due to a faster turnover of ODC in *T. b. rhodesiense*

compared to *T. b. gambiense* (53, 54, 61). Although the drug has a much better safely profile than melarsoprol, eflornithine treatment still leads to adverse reactions, such as nausea, vomiting, diarrhea, seizures, fever, convulsions and bone marrow toxicity causing anemia, leucopenia and thrombocytopenia (8). Eflornithine therapy is expensive and complex to administer. These factors keep the drug from wider use as first line treatment replacing melarsoprol for late stage HAT. Development of resistance is also a concern with eflornithine monotherapy. Combination therapy (described below) should help to protect the efficacy of eflornithine.

1.2.2.3 Nifurtimox and nifurtimox-eflornithine combination therapy (NECT)

Nifurtimox, introduced for the treatment of Chagas disease (*Trypanosoma cruzi* infection) in the 1960s, was registered to treat HAT in combination with eflornithine in 2009 (139). The precise mode of action of nifurtimox on *T. cruzi* remains unclear. The drug was suggested to act by inducing oxidative stress based on the observation of reactive oxygen and nitrogen species in nifurtimox-treated parasites or extracts (31-33). On the other hand, an alternative mechanism was raised in 2011 proposing that nifurtimox was metabolized by a type I nitroreductase to generate a toxic metabolite (45). Although the metabolite is proposed to be equally toxic to host cells and parasites, trypanosomes are selectively killed by nifurtimox due to the expression of a parasite-encoded type I nitroreductase.

NECT is used in late stage *T. b. gambiense* HAT. Compared with eflornithine monotherapy, the advantage of NECT includes easier administration, shorter treatment duration, less severe drug-induced adverse effects, and lower cost. NECT therapy also reduced the chance of the development of resistance to eflornithine. However, administration of NECT is still complicated and its efficacy towards *T. b. rhodesiense* is questionable (11, 139).

1.3 CURRENT DRUG DISCOVERY EFFORTS

The current options to treat HAT are limited to monotherapy using the four drugs mentioned above along with NECT. These therapeutic approaches are far from satisfactory due to severe adverse effects, complex administration, acquired resistance, rising failure rates, and lack of availability (8, 11, 139). Besides these issues, there is not a single agent that is effective against both *T. b. rhodesiense* and *T. b. gambiense* infection and/or against both first and second stage of HAT. Thus, new drugs are needed against HAT with the following features (139): 1) a good safety profile; 2) easy administration, particularly by the oral route; 3) higher efficacy; 4) activity against both stage of the disease; 5) effectiveness against both subspecies of *T. brucei* and 6) affordability.

The increasing epidemic of HAT at the end of the 20th century provoked an increased interest in the discovery of new drug targets and lead identification and optimization for

HAT. The trypanosomal pathways studied as targets for new drugs include polyamine biosynthesis, fatty acid biosynthesis and function, glycolytic enzymes, trypanothione biosynthesis and utilization, purine and pyrimidine metabolism, DNA modification, tubulin assembly, kinases and proteases (7, 55). On the other hand, new leads were also identified from screening with whole cell *T. brucei* viability assays.

1.3.1 Diamidines

Pentamidine, a diamidine derivative, has been used in HAT treatment for over 70 years. As mentioned earlier, the reagent has several disadvantages including a lack of efficacy against second stage disease, complex injection for administration and severe adverse effects. The Consortium for Parasitic Drug Development (CPDD) has extensively explored aromatic diamidines for the discovery and development of potential new drugs with better profiles. DB289 (pafuramidine maleate, Figure 1.8) was discovered as an orally active prodrug of the diamidine DB75 (furamidine, Figure 1.8) (87). Oral administration of DB289 (Figure 1.8) resulted in equal efficacy and lower toxicity compared with intramuscular administration of pentamidine in a phase III clinical trial (7). However, clinical development of DB289 was discontinued due to unexpected renal toxicity. On the other hand, DB829 (Figure 1.8) with pyridyl-amidines replacing the benzamidine moieties present in DB289, showed good antitrypanosomal activity and the ability to cross the blood-brain barrier. This compound is considered for entry into

clinical development against second stage HAT; DB868 is the orally active prodrug of DB829.

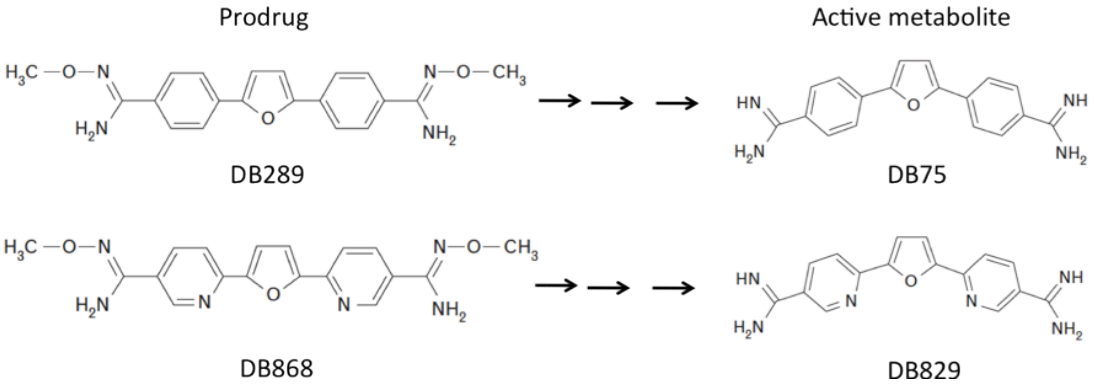
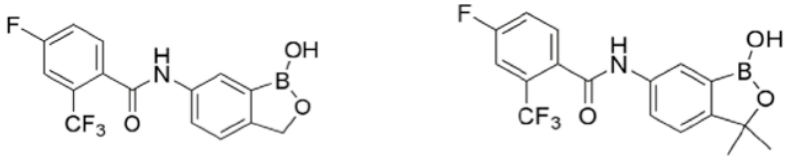


Figure 1.8 Chemical structures of antitrypanosomal diamidines and diamidine prodrugs.

1.3.2 Benzoxaboroles

The Drugs for Neglected Diseases Initiative (DNDi) has collaborated with Scynexis, Inc. to develop new drugs for second stage HAT. A whole cell *T. brucei* viability assay was utilized to screen a library of benzoxaboroles from Anacor Pharmaceuticals Inc. Benzoxaborole 6-carboxamides were identified as attractive starting points (57). With sufficient potency, desirable pharmacokinetic properties and acceptable blood-brain barrier permeability, SCYX-6759 (Figure 1.9) was selected as the initial lead. This compound completely cured infection in a stage 2 murine model of HAT. To optimize

the pharmacokinetics of SCYX-6759 for improved brain exposure without losing antitrypanosomal potency, SCYX-7158 was ultimately selected for progression to preclinical and phase I clinical studies (56). The mode of action of these benzoxaboroles remains unknown.



SCYX-6759 SCYX-7158

Figure 1.9 Chemical structures of benzoxaboroles.

1.3.3 Agents potentially leading to oxidative stress

Trypanosomatids have a peculiar ROS-scavenging system based on trypanothione-dependent peroxidases (58, 65, 66, 75, 106, 124). The biosynthesis and utilization of trypanothione in the parasite antioxidant defense system is shown in Figure 1.10. Lacking glutathione reductase, thioredoxin reductase, catalase and selenium-containing glutathione peroxidases possibly makes the trypanosomatids deficient in defense against oxidative stress. ROS generation has been widely studied as a mechanism for drugs targeting *T. cruzi*. In fact, the two agents used clinically for treating American

trypanosomiasis, nifurtimox and benznidazole, are both suggested to induce free radical formation (31-33, 75). Enzymes involved in trypanothione biosynthesis and utilization pathways have been shown to be essential to cell growth through the use of RNAi-mediated gene downregulation. Among these enzymes, trypanothione synthetase and trypanothione reductase have been extensively studied as drug targets against HAT (4, 20, 90, 92, 121, 138). Also, the inhibition of essential enzymes in the polyamine biosynthesis pathway, such as ODC and S-adenosylmethionine decarboxylase, could possibly cause oxidative stress, as spermidine is the precursor in the trypanothione biosynthesis pathway (35).

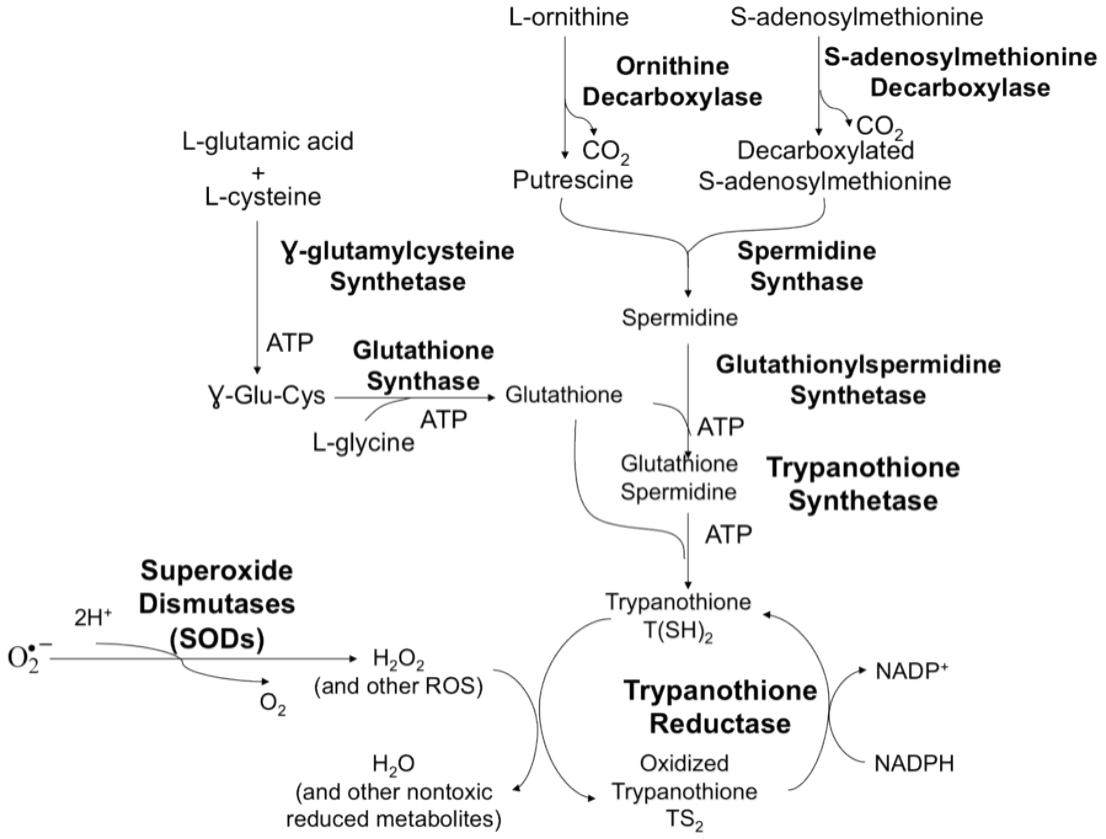


Figure 1.10 Biosynthesis and utilization of trypanothione in the trypanosomal antioxidant defense system.

1.3.3.1 Trypanothione synthetase inhibitors

Genetic and chemical validation of trypanothione synthetase (TbTryS) as a drug target has been reported by Fairlamb *et al.* (4, 20, 90, 92, 121, 138). High throughput screening was utilized to find inhibitors of trypanothione synthetase, and several hits series were

identified (Figure 1.11). The lead compounds possessed good target selectivity against TbTryS but relatively weak activity (5-30 μM EC_{50} values) in parasite proliferation assay (118).

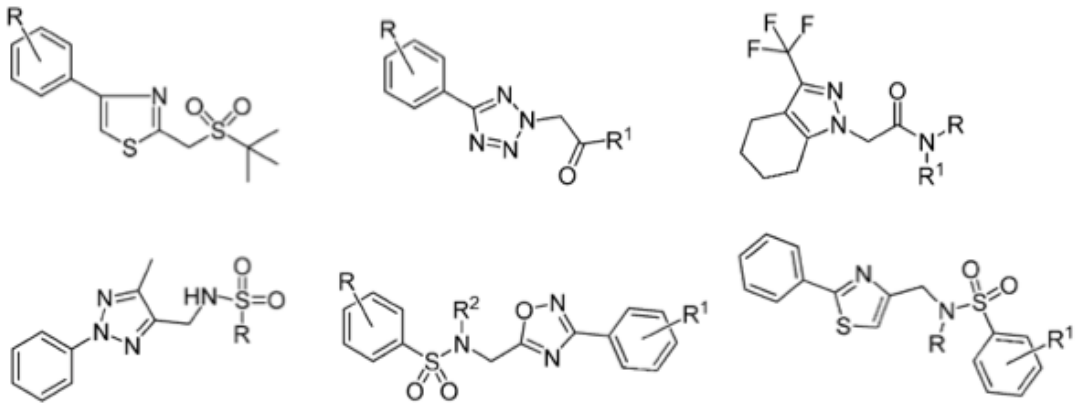


Figure 1.11 Clusters of hits identified in the high throughput TryS assays.

1.3.3.2 Trypanothione reductase inhibitors

Trypanothione reductase (TryR) has also been genetically validated as drug target by Fairlamb *et al.* (90, 92). Two series of drug-like TryR inhibitors, dihydroquinazolines and indatralines, were recently identified using structure-based and ligand-based drug design respectively (90, 129) (Figure 1.12). Lead compounds from both series showed moderate potency against TryR and *T. brucei* parasite growth. There was not significant correlation between the trypanocidal activity of these compounds and their TryR inhibition activity,

however. Further optimizations are required for these series to improve their potency and selectivity.

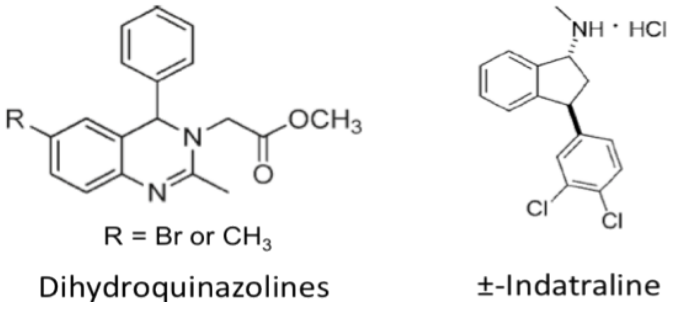


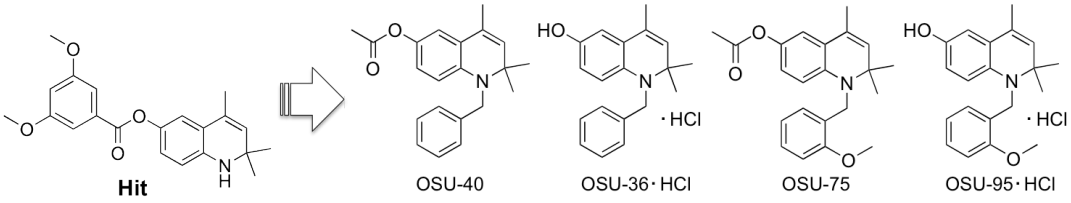
Figure 1.12 Chemical structures of TryR inhibitors.

1.4. DISCOVERY OF ANTITRYPANOSOMAL DIHYDROQUINOLINES

Sponsored by WHO's Special Programme from Research and Training in Tropical Diseases (WHO/TDR), a whole-cell screening program on compounds from Specs (www.specs.net) was carried out by Tibotec Belgium and the Swiss Tropical Institiute (STI). A hit compound containing the dihydroquinoline core structure was identified with potent in vitro activity against *T. b. rhodesiense* (Table 1.1) but no potency in vivo.

Based on this initial hit, a series of 1,2-dihydroquinolines were prepared, characterized and evaluated by Fotie *et al.* for their in vitro and in vivo activity against *T. brucei*

infection. From this medicinal chemistry investigation, several N1-substituted 1,2-dihydroquinoline-6-ols were discovered displaying nanomolar IC_{50} values *in vitro* against *T. b. rhodesiense* and selectivity indexes (SI) up to >18,000 (39). OSU-40 (1-benzyl-1,2-dihydro-2,2,4-trimethylquinolin-6-yl acetate, Table 1.1) showed a potency (IC_{50} = 0.014 μM, SI = 1,700) close to that of melarsoprol (IC_{50} = 0.008 μM, SI = 1,000) against *T. b. rhodesiense* STIB900 *in vitro*. In an early treatment acute mouse model of African trypanosomiasis, OSU-40 prolonged the lifespan of infected mice when given ip at 50 mg/kg/day for four consecutive days (>14 days vs. 7.75 days for untreated controls). A subsequent study showed that OSU-36•HCl, OSU-75 and OSU-95•HCl (Table 1.1) provided cures in a murine model of African trypanosomiasis when given ip at 50 mg/kg/day for four days (99).



Compound	IC_{50} (μM)		Selectivity Index	Dose (mg/kg)/ day	In vivo activity against *T. b. brucei* in Mice	In vivo activity against *T. b. rhodesiense* in Mice
	T. b. rhodesiense	L6 rat myoblasts			Mean survival days	Cure/treated*
Hit	0.054	16	300	4 × 50, ip	No prolongation/Cure	---
OSU-40	0.014	24	1710	4 × 50, ip	>14 from 7.75	0/4
OSU-36•HCl	0.013	11	850	4 × 50, ip	---	4/4
OSU-75	0.011	17	1550	4 × 50, ip	---	4/4
OSU-95•HCl	0.014	12	860	4 × 50, ip	---	4/4

Table 1.1 In vitro and in vivo potency and cytotoxicity of representative dihydroquinolines.

Overall, we have observed excellent antitrypanosomal activity both in vitro and in vivo with this series of 1,2-dihydroquinolines. The ultimate goal of our work is to develop a new drug based on these compounds against HAT. As discussed earlier in this chapter, the drugs currently used in HAT chemotherapy have serious limitations, including complex administration, severe toxicity, poor efficacy, acquired resistance, rising failure rates, and lack of availability (8, 11, 139). Accordingly, drug discovery efforts for HAT treatment need to focus on new reagents with the following characteristics: safety, affordability, ease of administration, activity against first and second stage disease, and effectiveness against both subspecies of *T. brucei* (11, 139). Thus, a series of studies were

carried out with representative dihydroquinolines to evaluate their mode of action and drug-like properties to aid the development of these compounds as prospective new HAT treatments. Chapter 2 and chapter 3 are focused on the methods, results and discussion of these studies.

CHAPTER 2: MODE OF ANTITRYPANOSOMAL ACTION OF DIHYDROQUINOLINES

For drugs and drug candidates, knowledge of their mode of action can direct efforts to improve activity, pharmaceutical properties, and clinical applications. Mode of action studies can also shed light on the basis for selectivity and mechanisms of resistance. Understanding the mode of action of a novel series of compounds should also provide direction for further structural modifications to improve activity.

The following observations pointed to induction of oxidative stress as a mechanism to account for the antitrypanosomal activity of the dihydroquinolin-6-ols. The structure-activity relationship of the dihydroquinolines suggested that only compounds with a 6-oxygen atom substituent displayed potent *in vitro* antitrypanosomal activity (39). An experiment using the redox-sensitive dye CM-H_2DCFDA was consistent with the hypothesis that ROS were produced in the parasites dose-dependently when *T. b. brucei* was exposed to OSU-40 *in vitro* (39).

2.1 AIMS OF STUDY

In this study we used OSU-40 to further investigate the mode of action of these promising dihydroquinolines. RNA interference (RNAi) of enzymes involved in parasite antioxidant defense and ESR experiments were conducted to provide support for the hypothesis that OSU-40 induces oxidative stress. These studies suggested the induction of oxidative stress as a promising target for anti-HAT drug development and may open the door for new therapeutic strategies against African trypanosomiasis.

2.2 MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich Chemical Co. unless stated otherwise. OSU-40 was synthesized following the procedure of Fotie *et al*. (39).

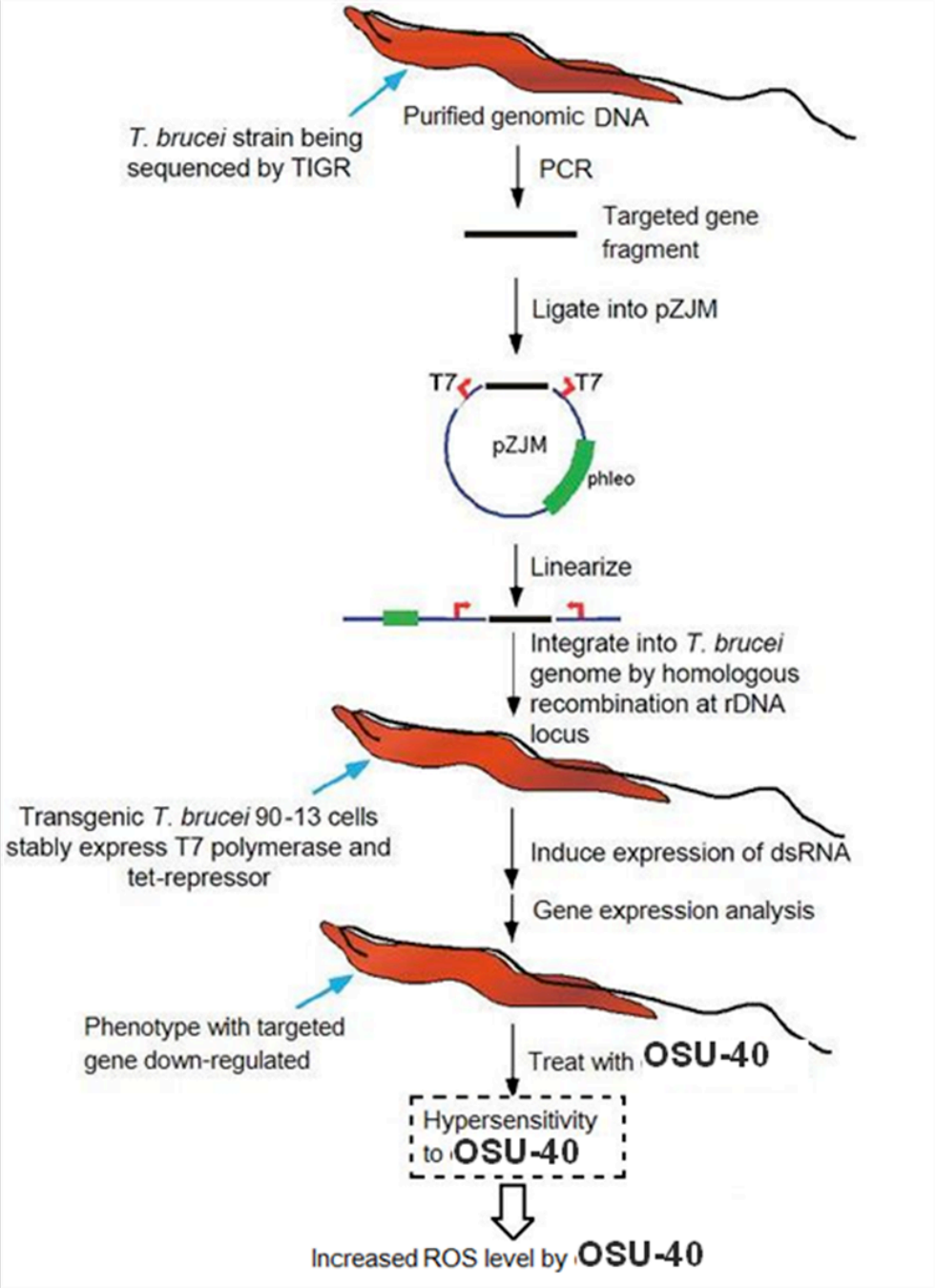


Figure 2.1 Summary of RNAi study.

2.1.1 Generation of RNAi parasites

2.1.1.1 Trypanosome culture

Bloodstream form *T. brucei brucei* strain 221 and transgenic cell line 90-13 (a gift from Dr. James Morris, Clemson University, SC, U.S.A.) were cultured at 37°C under 5% CO_2 in HMI-9 medium (50) supplemented with 20% fetal bovine serum. 90-13 cells were supplemented with 2.5 μg/ml G418 and 5 μg/ml hygromycin.

2.1.1.2 Generation of RNAi constructs

Fragments of the *T. brucei* genes encoding trypanothione synthetase (TbTryS) and superoxide dismutase B (TbSODB) were amplified by PCR from *T. b. brucei* 90-13 genomic DNA (see Table 1 for primer sequences), digested with HindIII/XhoI and cloned into the pZJMα vector (130) replacing the α-tubulin stuffer region, and transformed into DH5α™ competent cells (Invitrogen, Carlsbad, CA). Plasmid DNA was purified using a DNA maxi-prep kit (Qiagen, Valencia, CA) and inserts were verified by DNA sequencing. Prior to transfection, the plasmids pZJMα-TbTRYS and pZJMα-TbSODB were linearized by digestion with NotI.

2.1.1.3 Transfection of the parent cell line

Bloodstream form parental 90-13 cells were transfected with the Nucleofector® system (Lonza, Basel, Switzerland). A total of 1.5-3 × 10^7 cells at mid-log phase (culture density: 3-6 × 10^5 cells/ml) were resuspended in 100 µl of freshly-made human T-cell Nucleofector® solution, mixed with 5 µg of the desired linearized pZJMα construct, and subjected to nucleofection employing program X-001 (10). Immediately after transfection, cells were transferred into 75 ml of pre-warmed medium and incubated overnight for recovery at 37°C under 5% CO_2. Selection was achieved by the addition of 2.5 µg/ml phleomycin. For controls, the same procedure was conducted without DNA. Resistant clones were evident after approximately 10 days and transferred to culture flasks. Clonal cells were obtained by limiting dilution.

2.1.2 Characterization of RNAi phenotype parasites

2.1.2.1 Phenotype growth inhibition

Bloodstream form trypanosomes transfected with each of the RNAi constructs were maintained at cell densities from 1 × 10^5-5 × 10^6 cells/ml at 37°C in the presence or absence of 1 µg/ml tetracycline. Every 20-24 hrs, aliquots were taken from cell culture and parasite growth was assessed microscopically. Cumulative cell density was obtained by multiplying the cell density by the overall dilution factor at each time point.

2.1.2.2 Quantitative real-time PCR (qRT-PCR)

Approximately 0.5-1 × 10^7 cells were collected three days after the induction of RNAi, and RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). Residual DNA was removed using the TURBO DNA-*free*™ Kit (Applied Biosystems, Carlsbad, CA). cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). qRT-PCR was performed using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) in a Bio-Rad CFX96™ real-time PCR system (initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation (94°C, 10 s), and annealing and extension (57°C, 30 s)). All reactions were normalized to trypanosome β-actin (TbActin). Relative mRNA expression was analyzed using the $2^{-\Delta\Delta CT}$ method (70). Primer sequences for TbTryS and TbActin are shown in Table 2.1.

Usage	Gene product	Sequence of[a]: Forward primer	Reverse primer
RNAi construct	TbTryS	ctcgagCACGTTCCCTTTGGTGAGAT	aagcttCTCTGCTGCATGATCCTCAA
	TbSODB	ctcgagCAAAGGGCATATCGAAGGAA	aagcttGCTTGAGATCCGCTTCAGTC
qRT-PCR	TbTryS	CCATTGGTGTTGCATGAGTC	TGTCAAGTCCAGCCAGTCAG
	TbActin	GCCACGTATTTCCATCCATC	CCTGAGCTTCATCACCAACA

[a] Lowercase letters represent XhoI and HindIII sites.

Table 2.1 Primer sequences for RNAi constructs and qRT-PCR.

2.1.2.3 Western blotting

All steps were carried out at room temperature unless otherwise noted. Approximately 1-1.5 × 10^7 cells were pelleted by centrifugation at 800 × *g* for 10 min and lysed in 30-50 µl of lysis buffer containing 50 mM Tris-HCl, pH 8.1, 1% sodium dodecyl sulfate (SDS), 10 mM EDTA and SIGMAFAST protease inhibitor tablets. Lysates were sonicated for 10 s using a Virtis Virsonic 300 ultrasonic cell disruptor and cooled on ice for 10 s. Cell debris was cleared by centrifugation at 13,200 × *g*, 15 min, 4°C. Protein concentrations of soluble fractions were measured using a bicinchoninic acid assay (Pierce, Rockford, IL). Lysates were boiled for 10 min following addition of 5 × SDS-polyacrylamide gel electrophoresis sample buffer (300 mM Tris-HCl, pH 6.8, 10% SDS, 5% β-mercaptoethanol, 50% glycerol and 0.05% bromophenol blue). Ten µg of protein from each lysate was resolved on 10% SDS-polyacrylamide gels and transferred to Amersham™ Hybond™-ECL nitrocellulose membrane (GE Healthcare, Pittsburgh, PA). Membranes were blocked with 5% (w/v) non-fat milk in TBST (20 mM Tris-HCl, 150 mM NaCl, pH 7.6, 0.1% Tween 20) for 30 min and washed 3 × 10 min with TBST prior to incubation with primary antiserum. Rat polyclonal antiserum against recombinant TbTryS (provided by Dr. Alan Fairlamb, University of Dundee, UK) was diluted 1:500 in TBST containing 5% bovine serum albumin (w/v). Rabbit polyclonal antiserum against parasite α-enolase (provided by Dr. Paul Michels, Catholic University of Louvain, Brussels, Belgium) was diluted 1:1000 in TBST containing 5% (w/v) non-fat milk. The

membranes were washed 3 × 10 min in TBST and incubated with goat anti-rat or anti-rabbit immunoglobulin G-horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc.) conjugates (1:5000) for 1 h. Following 3 × 10 min washes with TBST, the proteins were visualized by chemiluminescence with Amersham^{TM} ECL^{TM} western blotting detection reagent (GE Healthcare, UK).

2.1.3 In vitro susceptibility assay

All incubations were carried out at 37°C in a humidified 5% CO_2 atmosphere, unless otherwise noted. The activity of compounds towards TbTryS-RNAi and TbSODB-RNAi cell lines were tested in the presence or absence of 1 μg/ml tetracycline following the procedure of Reid *et al*. (99) with some modifications. Parasite cultures at a starting density of 1×10^5 cells/ml were seeded in 96-well plates in a volume of 100 μl/well with or without test compounds for 72 h. Twenty-five μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml in autoclaved water) was added to each well, followed by an additional 2 h incubation. One hundred μl of 10% SDS lysis buffer (prepared in 50% dimethyl formamide) was added to each well and plates were incubated for 3-6 hrs. A SpectraMax Plus microplate reader (Amersham Biosciences, Piscataway, NJ) was used to measure the optical densities of each well at 570 nm. IC_{50} values were determined with the aid of the software program SoftMax Pro (Amersham Biosciences, Piscataway, NJ) using the dose-response equation $y = [(a - d)/(1 + (x/c)^b)] + d$, where $x =$

the drug concentration, y = absorbance at 570 nm, a = upper asymptote, b = slope, c = IC_{50}, and d = lower asymptote.

2.1.4 Electron spin resonance (ESR)

The production of free radicals was detected by ESR after incubation of compounds with BSF *T. b. brucei* strain 221 parasites. Cells in late log stage were centrifuged at 800 × *g* for 10 min and resuspended in 50 mM phosphate buffer (pH 7.4) in the presence of 10 mM glucose, 10 mM spin trapping reagent 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO) and 10 μM chelating reagent diethylenetriamine-pentaacetic acid (DETPAC). Stock solutions of the compounds in DMSO were added, after which the suspension was transferred into a 50 μl capillary tube and incubated for 60 min. ESR spectra were recorded on a Bruker EMX X-band spectrometer system (Bruker Biospin, Billerica, MA) and characterized by computer simulations using Winsim software (Public EPR Software Tools, National Institute of Environmental Health Sciences, Durham, NC).

2.2 RESULTS AND DISCUSSION

2.2.1 Effect of RNAi-mediated knockdown of TbTryS

To assess the role of TbTryS in *T. brucei* sensitivity to OSU-40, bloodstream form 90-13 cells were transfected with plasmid pZJMα-TbTRYS. This allows induction of TbTryS

RNAi in the presence of tetracycline and consequent reduction in TbTryS expression. In the absence of tetracycline, TbTryS-RNAi cells grew at approximately the same rate as the parental 90-13 cells (data not shown). Following the addition of tetracycline for 72 h, a significant reduction in the growth rate of TbTryS-RNAi cells was observed with a corresponding 4-fold reduction of endogenous TbTryS mRNA (Fig 2.1A). The doubling time increased from 8.6 h to 11.1 h as measured over the first 5 days post induction with a cumulative cell density for induced parasites being 11% of the non-induced cultures on day 5. Western blot analysis confirmed that the level of TbTryS protein was significantly reduced in TbTryS-RNAi-induced cells compared to non-induced and wild-type cultures (Fig 2.1B). Results above were consistent with reported effects of TbTryS RNAi on parasite growth and the level of TbTryS protein in *T. brucei* parasites (4, 20).

TbTryS is an essential enzyme in the parasite defense against oxidative stress (4, 20). If the trypanocidal effect of OSU-40 is mediated through extensive oxidative stress, we hypothesized that down-regulation of TbTryS should then sensitize the parasites to OSU-40. Consistent with our hypothesis, RNAi-induced BSF trypanosomes were 2.4-fold ($P < 0.05$) more susceptible to OSU-40 as determined by MTT assays of parasite viability (Fig 2C). H_2O_2, nifurtimox and suramin were employed as controls. Both H_2O_2 and nifurtimox were reported to show increased activity on trypanosomes with TbTryS knockdown by RNAi (4, 20). Suramin, which served as a negative control for the changes in potency of OSU-40 following TbTryS-knockdown, acts through a mechanism not directly related to oxidative stress (8). The potencies of H_2O_2 and nifurtimox both

increased on RNAi-induced parasites by 2.2-fold ($P < 0.01$) and 1.6-fold ($P < 0.05$) respectively, while the activity of suramin was unchanged.

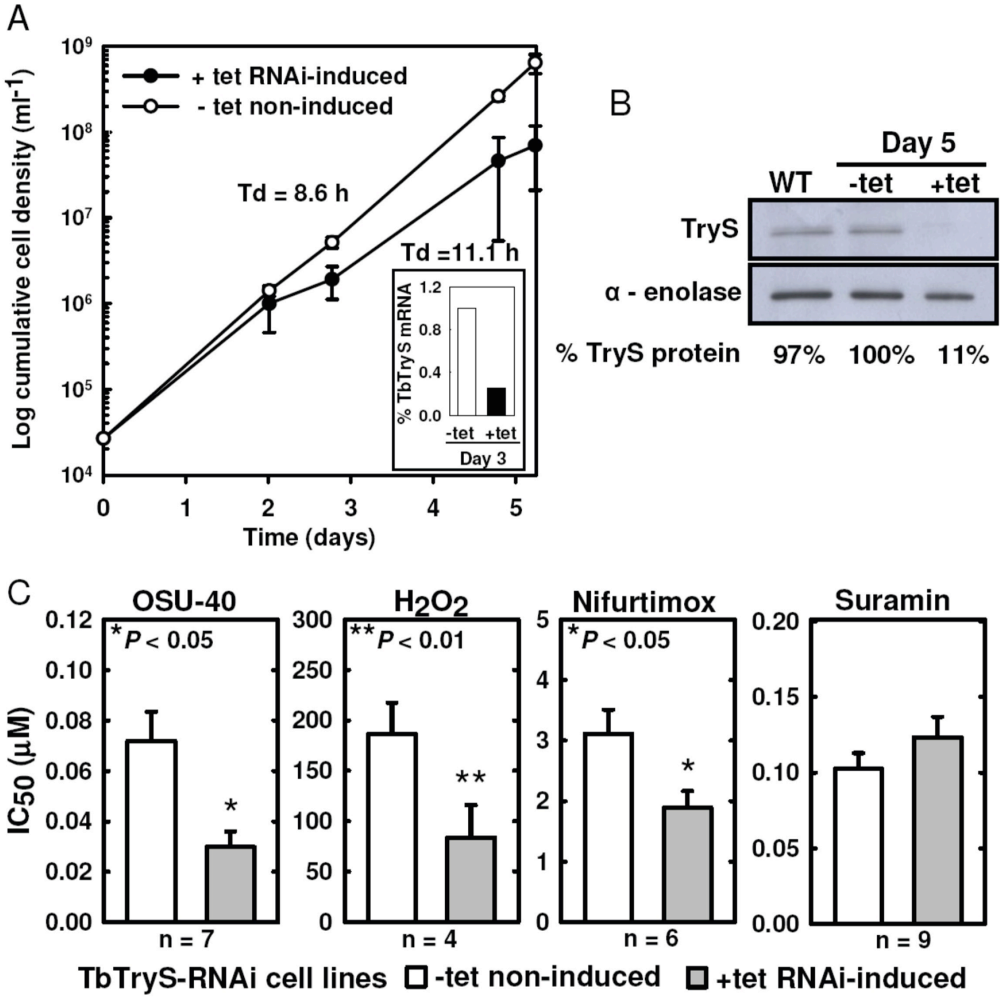


Figure 2.2 Effect of TbTryS RNAi on bloodstream form parasite growth, TbTryS protein level and susceptibility to OSU-40.

A, growth of RNAi-induced (+tet) and non-induced (-tet) TbTryS-RNAi cell lines. 'Td' indicates the doubling time over 5 days; Results are means ± SE from three independent experiments. **Inset of A,** real-time PCR shows 4-fold lower TryS mRNA levels in RNAi-induced (+tet) compared to noninduced (-tet) cells collected three days after induction. **B,** Western blot analysis of TbTryS expression in wild-type (WT) 90-13, non-induced (-tet) and RNAi-induced (+tet) trypanosomes collected 5 days after induction. %TbTryS indicates protein abundance relative to non-induced cells quantified using ImageJ software (public domain, National Institutes of Health) after normalizing to α-enolase bands. The blot is representative of three independent experiments. **C,** Susceptibility of RNAi-induced (+tet) and noninduced (-tet) TbTryS-RNAi cell lines to OSU-40, H_2O_2, nifurtimox and suramin. RNAi was initiated and maintained for at least three days prior to initiation of the susceptibility assay (see Materials and Methods). The results shown are the means of at least four independent experiments ± SE (n = the number of independent experiments). Asterisks (*$P < 0.05$ and **$P < 0.01$) indicate statistically significant differences in IC_{50} values between RNAi-induced and non-induced cells as assessed by Student's *t* test.

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2.2.2 Effect of RNAi-mediated knockdown of TbSODB

To further confirm that down-regulation of essential trypanosome antioxidant enzymes leads to an increased sensitivity to OSU-40, a TbSODB-RNAi cell line was also generated. Consistent with previously reported findings (136), RNAi-mediated down-regulation of TbSODB led to a dramatic decrease in parasite growth rate (Fig 2.2A), with the doubling time lengthened from 5.7 h to 9.3 h over the first 5 days after induction and the cumulative cell density of induced parasites being as low as 0.3% of the non-induced cultures on day 5.

The antiparasitic potency of OSU-40 was evaluated in TbSODB-RNAi-induced and non-induced parasites, together with H_2O_2, nifurtimox and suramin. Similar to our observation with TbTryS-RNAi-induced trypanosomes, parasites with down-regulated TbSODB became 3.4-fold ($P < 0.01$) more susceptible to OSU-40. H_2O_2 and nifurtimox were also more active on RNAi-induced parasites (2.6-fold ($P < 0.05$) and 4.1-fold ($P<0.005$), respectively). At the same time, down-regulation of TbSODB did not lead to a significant rise in susceptibility to suramin. This again confirmed that the increased activity of OSU-40 was not a general effect due to the knockdown of TbSODB.

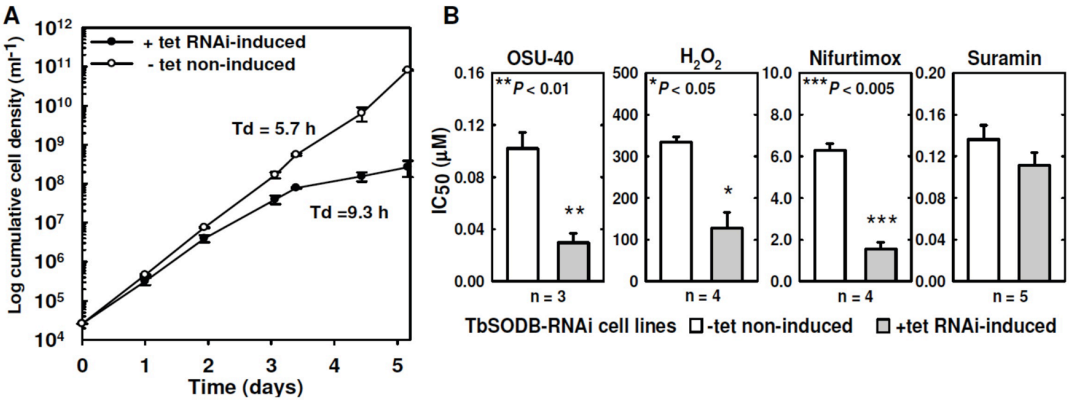

Figure 2.3 Effect of TbSODB RNAi on parasite growth and susceptibility to OSU-40. **A,** growth of RNAi-induced (+tet) and non-induced (-tet) TbSODB-RNAi cell lines. 'Td' indicates the doubling time over 5 days. The results are means ± SE from two independent experiments. **B,** Susceptibility of RNAi-induced (+tet) and noninduced (-tet) TbSODB-RNAi cell lines to OSU-40, H_2O_2, nifurtimox and suramin. RNAi was initiated and maintained for at least 2 days prior to susceptibility assays. The results shown are the means of at least three independent experiments ± SE (n = the number of independent experiments). Asterisks (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$) indicate statistically significant differences in IC_{50} values between RNAi-induced and non-induced cells as assessed by Student's *t* test.

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We observed a more dramatic increase in susceptibility to OSU-40 in TbSODB-RNAi parasites (3.4-fold) compared to the results with TbTryS-RNAi parasites (2.4-fold). Reduced expression of TbSODB would be expected to lead to rapid accumulation of superoxide anion within the trypanosome, considering that TbSODs are the only enzymes responsible for detoxifying this highly toxic radical in the parasite. However, RNAi-mediated knockdown of TbTryS was previously reported to increase expression of trypanothione reductase (TbTryR), γ – glutamylcysteine synthetase (Tbγ – GCS), and ornithine decarboxylase (TbODC) (4). It is possible that the upregulation of these enzymes could serve to maintain a favorable intracellular redox potential and lead to less dramatic effects on both growth rate and OSU-40 susceptibility in TbTryS-RNAi-induced parasites. In addition to work with TbTryS and TbSODB, we generated parasites harboring a TbSODA RNAi targeting construct. While 6-fold lower mRNA levels were observed three days after the addition of tetracycline, neither the growth rate nor the susceptibility to OSU-40 was affected in TbSODA-RNAi organisms (data not shown). A possible explanation for these observations may be that TbSODB could compensate for the loss of TbSODA in the TbSODA-RNAi induced parasites.

2.2.3 ESR study

Parasites were treated with OSU-40 for 1 h and the cell suspension was analyzed using ESR spectroscopy. The spin trapping reagent DEPMPO was added to the cell suspension to permit the measurement of short-lived free radicals. Clearly resolved 12 line spectra

were observed after treatment of trypanosomes with both OSU-40 and H_2O_2 (Fig 2.3 A). Computer simulation was carried out to characterize the ESR spectra obtained after exposure of parasites to 10 μM OSU-40 for 60 min. The simulation identified the presence of two isomers of DEPMPO adduct with the following coupling constants (G): isomer 1: ^{31}P, 47.049; ^{1}H, 20.033; ^{14}N 14.494 and isomer 2: ^{31}P, 46.402; ^{1}H, 22.398; ^{14}N 14.445. The calculated spectral parameters were in agreement with that of carbon-centered adduct (127). Similar results were obtained for all the DEPMPO adducts following exposure of the parasites to OSU-40 and H_2O_2. Induction of free radicals by OSU-40 in BSF *T. brucei* was dose-dependent based on quantification of ESR signal amplitudes (Fig 2.3 B).

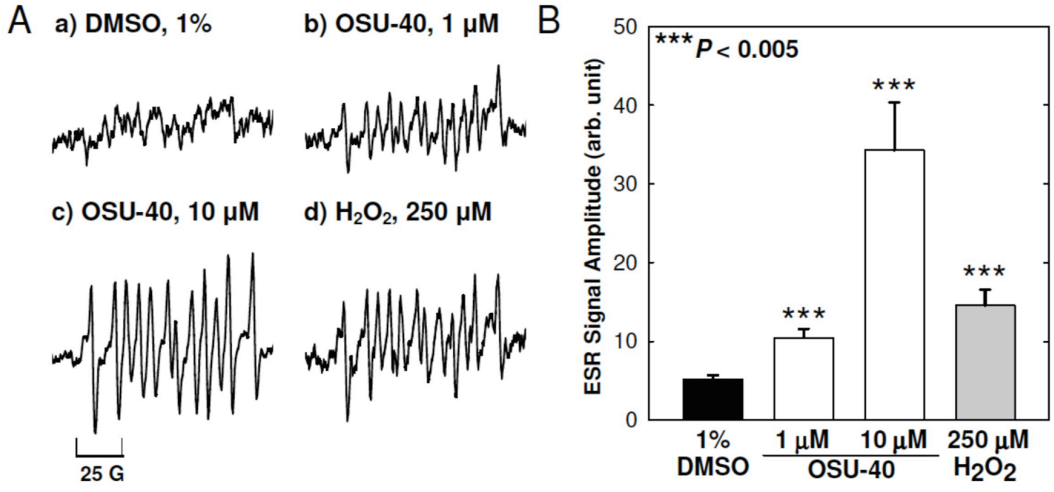

Figure 2.4 ESR spectra and signal quantification in trypanosomes exposed to OSU-40.

A, ESR spectra observed after incubation of bloodstream form *T. b. b.* 221 cells with a) DMSO, 1%; b) OSU-40, 1 μM; c) OSU-40, 10 μM and d) H_2O_2, 250 μM; **B,** quantification of ESR signals. Results are means ± SD from three independent experiments. Asterisks (***$P < 0.005$) indicate statistically significant differences between the DMSO-treated group and the treatment groups as assessed by Student's *t* test.

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We have also shown direct evidence through ESR spectroscopy that the dihydroquinoline OSU-40 induces free radical formation. ESR (also known as electron paramagnetic resonance (EPR)) detects paramagnetic species in an external magnetic field, where the paramagnetic species absorb microwave energy leading to the transition of spin states (67, 128). Spin trapping allows the detection of radicals by ESR through the formation of radical adducts between the spin trapping reagent and free radicals. The technology has been used in chemical systems and increasingly in biomedical applications, including the measurement of free radical formation in Trypanosomatids and other biological systems (16, 86, 94, 128). In this study, inclusion of 1 μM OSU-40 in trypanosome cultures resulted in a clear ESR signal in as little as 30 min (data not shown). Also, a dose-dependent production of signals corresponding to free radical species was observed after

treating the parasites with OSU-40 for 60 min. These data are consistent with our previous experiments showing increased fluorescence when trypanosomes were incubated with the redox active dye CM-H_2DCFDA in the presence of OSU-40 (39). The spectra recorded after treatment of trypanosomes with OSU-40 and H_2O_2 showed identical ESR features which suggest the radicals to be carbon-centered in both cases and possibly produced by the same mechanism. However, we do not know if this represents initial or end products and whether the initial products are different in the two cases.

2.3 SUMMARY AND CONCLUSIONS

In this chapter we present data that suggest the activity of antitrypanosomal dihydroquinolines is through induction of oxidative stress. We rationalize that the potency and selectivity of this series of compounds is based on the limited ROS-scavenging capacity of the parasite's redox system and/or peculiarities in the redox status of trypanosomes compared to host cells (58, 65, 66, 75, 106, 124).

We hypothesized that parasites with reduced expression of ROS detoxifying enzymes would offer the opportunity to test our idea that antitrypanosomal dihydroquinolines act through inducing oxidative stress. For these experiments we used an inducible RNAi system to independently knockdown the ROS detoxifying enzymes TbTryS and TbSODB, thus creating parasites with a compromised ROS-detoxification system. We selected these enzymes on the basis of their proven essentiality using both chemical and

genetic approaches (4, 20, 97, 121, 136, 138). TbTryS has been validated as a drug target using small molecule inhibitors (121) and TbSODB-null parasites show increased sensitivity to the superoxide generating molecules paraquat and nifurtimox (97). In our experiments, an increased susceptibility to OSU-40 was observed upon knockdown of either TbTryS or TbSODB, consistent with the hypothesis that antitrypanosomal dihydroquinolines induce oxidative stress in the parasite.

A pharmacokinetic study of OSU-40 and OSU-36·HCl in rats showed that OSU-40 was rapidly converted to OSU-36 in plasma, with OSU-36 subsequently undergoing oxidative metabolism (42). Together with the results obtained here, we propose the following addition to the proposed mode of action of antitrypanosomal dihydroquinolines (39) (Figure 2.4): Compounds esterified at the 6-oxygen atom are hydrolyzed by esterases and then oxidized to the corresponding quinone imine species spontaneously. Redox cycling between the quinone imine and semiquinone radical results in the generation of superoxide. Trypanothione reductase has been reported to catalyze the reduction of quinones (15, 48, 102) and could facilitate the initiation of single electron redox cycling. Hydrogen peroxide and hydroxyl radical formed as a consequence of superoxide production (52, 122, 124) together with superoxide would lead to *T. brucei* parasite cell death.

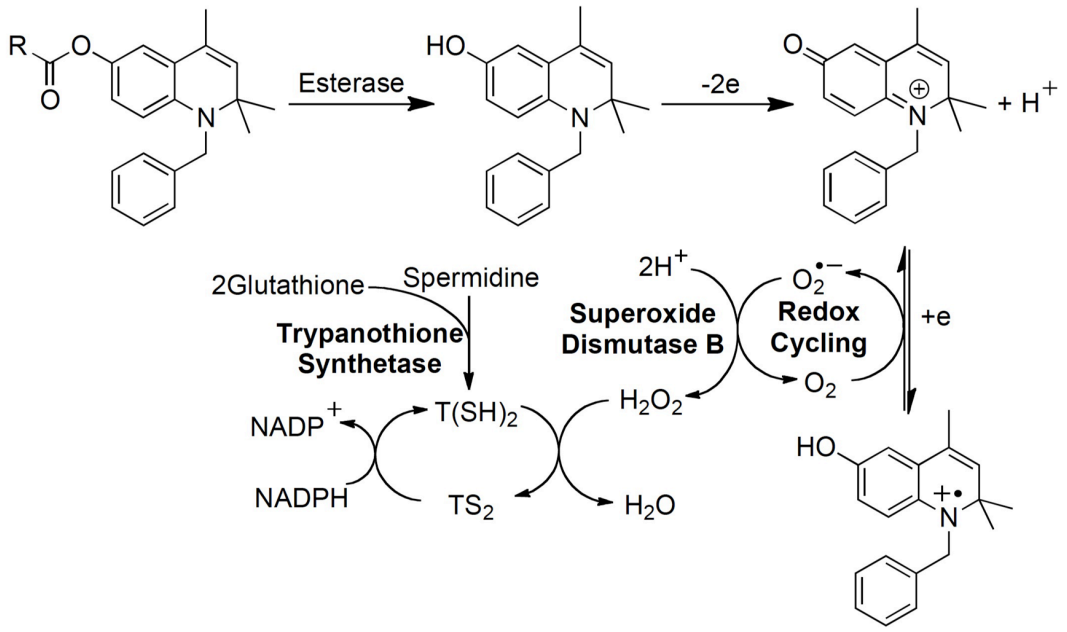

Figure 2.5 Proposed mode of action for the generation of oxidative stress by the antitrypanosomal dihydroquinolines including the possible protective role of TbTryS and TbSODB.

In this model, dihydroquinolines such as OSU-40 are hydrolyzed by an esterase activity followed by auto-oxidation. The resulting quinone imine can then undergo redox cycling, leading to oxidative stress. The results of this investigation suggest that the parasite's antioxidant enzymes trypanothione synthetase and superoxide dismutase B are utilized to lessen the toxic effects of this class of antitrypanosomal agents.

A central issue regarding reagents inducing oxidative stress is selectivity. Although increased levels of ROS are certainly toxic to a pathogen such as *T. brucei*, excessive amounts of these reactive species can be toxic to the host due to their high reactivity with biological molecules such as DNA, protein and lipids. However, selectivity can be achieved through different manipulation of vulnerability to oxidative stress. For example, targeting the ROS stress response has been proposed as a strategy to selectively kill cancer cells (98, 122, 126). The small molecule piperlongumine was recently reported to lead to apoptotic cell death in cancer cells by increasing the level of ROS (98). An altered redox status appears to make cancer cells more vulnerable to oxidative stress, providing the basis for selectivity of this novel chemotherapeutic strategy (98, 122, 126). In the case of trypanosomatids, ROS generation has been widely studied as a mechanism for drugs targeting *T. cruzi*. In fact, the two agents used clinically for treating American trypanosomiasis, nifurtimox and benznidazole, are both suggested to induce free radical formation (31-33, 75). The selectivity of ROS generating reagents for trypanosomatids over host cells might be explained by the unique biochemical features of these parasites, particularly their peculiar ROS-scavenging system based on trypanothione-dependent peroxidases (58, 65, 66, 75, 106, 124). Trypanosomatids also lack glutathione reductase, thioredoxin reductase, catalase and selenium-containing glutathione peroxidases, possibly making them deficient in defense against oxidative stress. Fairlamb *et al.* proposed that the essential parasite enzymes trypanothione synthetase (121, 138) and trypanothione

reductase (90, 92) are promising drug targets and reported different classes of small molecule inhibitors (90, 92, 121) interfering with the ROS-scavenging system. Taken together, these studies support the idea that the induction of oxidative stress can be an effective strategy for developing antitrypanosomal agents.

In conclusion, we have demonstrated that decreased expression of the key trypanosome antioxidant enzymes TbTryS and TbSODB results in an increased susceptibility of bloodstream form *T. brucei* to the representative dihydroquinoline OSU-40. We have also used ESR spectroscopy to directly detect the formation of free radicals upon exposure of *T. brucei* to OSU-40. These data strongly support that the mode of antitrypanosomal action for this class of compounds is to induce oxidative stress. Validation of the induction of oxidative stress as the mode of action for dihydroquinolines may open the door for new therapeutic strategies targeting African trypanosomiasis.

CHAPTER 3: AQUEOUS SOLUBILITY, CYTOTOXICITY AND IN VITRO RESISTANCE OF ANTITRYPANOSOMAL DIHYDROQUINOLINES

The ultimate goal of our work with antitrypanosomal dihydroquinolines is to develop a new drug against HAT. As discussed in chapter 1, the drugs currently used in HAT chemotherapy have serious limitations, including complex administration, severe toxicity, poor efficacy, acquired resistance, rising failure rates, and lack of availability (8, 11, 139). Accordingly, drug discovery efforts for HAT treatment need to focus on new reagents with the following characteristics: safety, affordability, ease of administration, activity against first and second stage disease, and effectiveness against both subspecies of *T. brucei* (11, 139). A series of studies were carried out with representative dihydroquinolines to evaluate their drug-like properties to aid the development of these compounds as prospective new HAT treatments.

3.1 AIMS OF STUDY

3.1.1 Aqueous solubility of dihydroquinolines

Complicated administration is a major disadvantage of all the current treatments for HAT, even for the newly registered NECT. Oral activity will simplify the administration, which is much desired for HAT treatment in rural endemic areas. Multiple factors can affect oral absorption and bioavailability, such as drug stability, solubility, metabolism and membrane permeability. Generally, high aqueous solubility and moderate lipophilicity can provide a better chance for good absorption and bioavailability (22, 69).

An evaluation of aqueous solubility can be carried out using either a thermodynamic assay or a kinetic assay. To obtain a thermodynamic solubility values, the solid compound of interest must be allowed to equilibrate with the liquid medium before quantification using the traditional thermodynamic assay (1). Measurements can be carried out by the shake-flask method, where the compound in a defined medium at a given pH and temperature will be shaken for a set period of time. The presence of insoluble compound will confirm the saturation of the supernatant. After filtration, analysis of the filtrate can be carried out with UV spectroscopy and chromatographic methods etc. (23). Although the traditional shake-flask method is believed to give the most accurate solubility value, it is not preferred in the early stage of drug discovery since it is time-consuming and requires relatively large quantities of the compound of interest. Recently, using a miniaturized shake-flask approach, a high throughput

equilibrium solubility assay has been reported as a fast, reliable, inexpensive screening method that is suitable for early-stage drug discovery (140).

Kinetic solubility assays, on the other hand, have been more widely utilized in early-stage lead optimization and candidate selection (19, 28, 38). The kinetic solubility value is determined on a non-equilibrium state and can be measured with turbidimetric/nephelometric methods or by UV absorption (23, 89). These assays are relatively fast, inexpensive and require small amounts of compounds when compared to the traditional shake-flask method. Also, DMSO solutions of compounds, the typical stock solution for in vitro and in vivo tests in early-stage drug discovery, are used in these measurements. This gives the kinetic methods another advantage, as they require less effort for sample preparation. Automated high throughput solubility assays have been reported for the measurement of kinetic aqueous solubility (19, 28, 38).

OSU-36•HCl and OSU-95•HCl are hydrochloride salts of dihydroquinolin-6-ols, while OSU-40 and OSU-75 are the methyl esters of their free bases, respectively (please see Table 1.1 for the structures of these compounds). In lead optimization of dihydroquinolines, one motivation for preparing these salts was to increase aqueous solubility. Thus, the aqueous solubility of OSU-40, OSU-36•HCl, OSU-75 and OSU-95•HCl was measured with a kinetic solubility assay, similar to those described in the literature (69, 89).

3.1.2 In vitro cytotoxicity to human liver cells

Reducing host toxicity is another goal for drug discovery for HAT. As concluded in chapter 2, the mode of antitrypanosomal action of dihydroquinolines is to induce oxidative stress through the formation of a quinone imine species. These species are also reported as metabolites of several drugs through cytochrome P450-catalyzed oxidation, including acetaminophen (123), diclofenac (120), thiabendazole (24) and lumiracoxib (62). The formation of quinone imine species is believed to cause hepatotoxicity. These electrophilic quinone imine species may covalently bind to proteins and potentially interfere with mitochondrial and nuclear function leading to reactive oxidative species and ultimately apoptosis in liver cells (49, 60, 78, 79, 95, 96, 123). Glutathione conjugated quinone amine intermediates were observed; the intermediates are believed to be products of the detoxification process of the quinone imine species (24, 62, 79, 120). However, glutathione conjugation also causes the depletion of cellular glutathione pools and consequentially impaired antioxidant function, leading to cell injury or death (24, 62, 79, 96, 120). As an example, cytochrome P450-catalyzed oxidation of acetaminophen to N-acetyl-p-benzoquinoneimine (NAPQI) and the following glutathione conjugation are shown in Figure 3.1 (59). Overall, the hepatotoxicity of the drugs mentioned above was suggested to depend on P450-catalyzed activation. As further support for this hypothesis, the toxic effects of acetaminophen within the liver were observed in the centrilobular zone, where the greatest concentration of a certain cytochrome P450 isoforms has been reported (46, 49, 123).

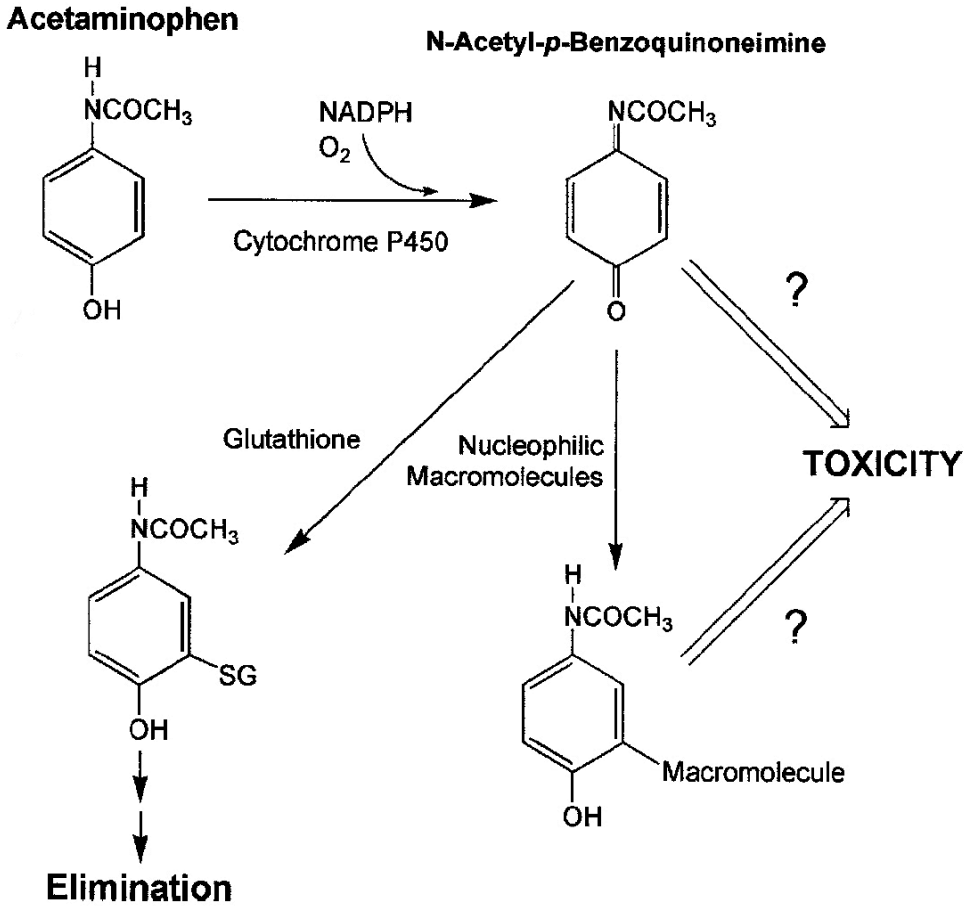

Figure 3.1 The role of metabolism of acetaminophen in its hepatotoxicity.

Acetaminophen is oxidized by cytochrome P450 to NAPQI and NAPQI may covalently bind to macromolecules, leading to toxicity. NAPQI-glutathione conjugation contributes to detoxification of acetaminophen.

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When preparing dihydroquinolines, we noticed that the analogues with a free 6'-OH substituent on the dihydroquinoline ring system turned to a dark, sticky gum within a few hours due to auto-oxidation (39). Hence, we hypothesized that the formation of a quinone imine species from antitrypanosomal dihydroqinolines was through auto-oxidation rather than oxidation catalyzed by a cytochrome P450 enzyme. Given that the hepatotoxicity of the drugs mentioned above was suggested to depend on P450-catalyzed activation, auto-oxidation of antitrypanosomal dihydroquinolines may not lead to toxicity that specifically targets liver cells. However, we cannot rule out the possibility of P450-catalyzed oxidation of dihydroquinolines to generate quinone imine species. To address the concern that dihydroquinolines may mediate host hepatotoxicity, the cytotoxicity of OSU-40 and OSU-36•HCl were measured against a panel of liver cell lines.

3.1.3 Mechanism of resistance of dihydroquinolines

Studies on the selection of nifurtimox-resistant *T. brucei* parasites have been reported to shed light on the activation mechanism of this drug (137) and possibility of cross-resistance to other drugs (116).

In our study, the selection of OSU-40-resistant *T. brucei* clones was achieved by exposing parasites in culture to long-term OSU-40 pressure. The susceptibility of parasites becoming increasingly resistant to OSU-40 was evaluated during the selection period (39), and *T. brucei* lines displaying resistance to dihydroquinolines were eventually developed. The analysis of OSU-40-resistant *T. brucei* clones may provide clues regarding the parasite target or targets for the trypanocidal activity of dihydroquinolines. In addition, the possibility of cross-resistance to current antitrypanosomal drugs can also be determined with OSU-40-resistant *T. brucei* clones.

3.2 MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich Chemical Co. unless stated otherwise. OSU-40, OSU-36•HCl, OSU-75 and OSU-95•HCl were synthesized following the procedures described by Fotie et al. (39) and Reid et al. (99).

3.2.1 Kinetic solubility assay

Compounds were dissolved and serially diluted in DMSO. Two μl of DMSO solution were added and mixed well in 100 μl of phosphate buffer (50 mM, pH = 7.4) in 96-well clear bottom white plates followed by the addition of another 100 μl of phosphate buffer (50 mM, pH = 7.4) with extensive mixing. The final concentration of DMSO is 1% (v/v)

in each well. The turbidity of each well was evaluated immediately by measuring the absorbance at 650 nM using a SpectraMax Plus microplate reader (Amersham Biosciences, Piscataway, NJ). Further advice regarding the implementation of this assay was provided by Dr. Robert Jacobs and his group at Scynexis Inc., Durham, NC.

3.2.2 Mammalian cell culture

HepG2, Hep3B, Huh7 and PLC5 cells (a gift from Dr. Chih-Shih Chen, Ohio State University, Columbus, OH) were maintained in 75 cm^2 culture flasks with Dulbecco's modification of Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin in 5% CO_2 at 37°C. The cells were trypsinized and passed every 2 – 4 days.

3.2.3 Liver cell cytotoxicity assay

All incubations were carried out at 37 °C in a humidified 5% CO_2 atmosphere. 5×10^3 cells were seeded and incubated overnight in DMEM medium supplemented with 10% fetal bovine serum in 96-well plates. Compounds in serial dilutions were added and incubated for 72 h in a volume of 100 μl/well. Twenty μl/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 2.5 mg/ml in autoclaved water) were added, and the plates were incubated for 1 h. Liquid was removed from each well and

100 µl/well of DMSO were added. A SpectraMax Plus microplate reader (Amersham Biosciences, Piscataway, NJ) was used to measure the optical densities of each well at 570 nm. IC_{50} values were determined with the aid of the software program SoftMax Pro.

3.2.4 Trypanosome culture

Bloodstream form *T. brucei brucei* strain 221 parasites were cultured at 37 °C under 5% CO_2 in HMI-9 medium (50) supplemented with 20% fetal bovine serum. Cell density was maintained between 2.5×10^4-5×10^6 cells/ml.

3.2.5 Generation of OSU-40-resistant *T. brucei* cell line

T. brucei brucei 221 parasites were cultured in the continuous presence of OSU-40. The concentration of OSU-40 in the culture was increased in a step-wise fashion, starting at 0.02 µM until the organisms were routinely cultured in 0.45 µM OSU-40. After approximately 22 months of exposure in OSU-40, clonal parasites were generated by limiting dilution.

3.2.6 Susceptibility of OSU-40-resistant Trypanosomes

To confirm resistance, the in vitro antitrypanosomal activity assay was performed after allowing the cells to grow in OSU-40-free conditions for 3 – 12 days. The assay was carried out following the same procedure described in section 2.1.3.

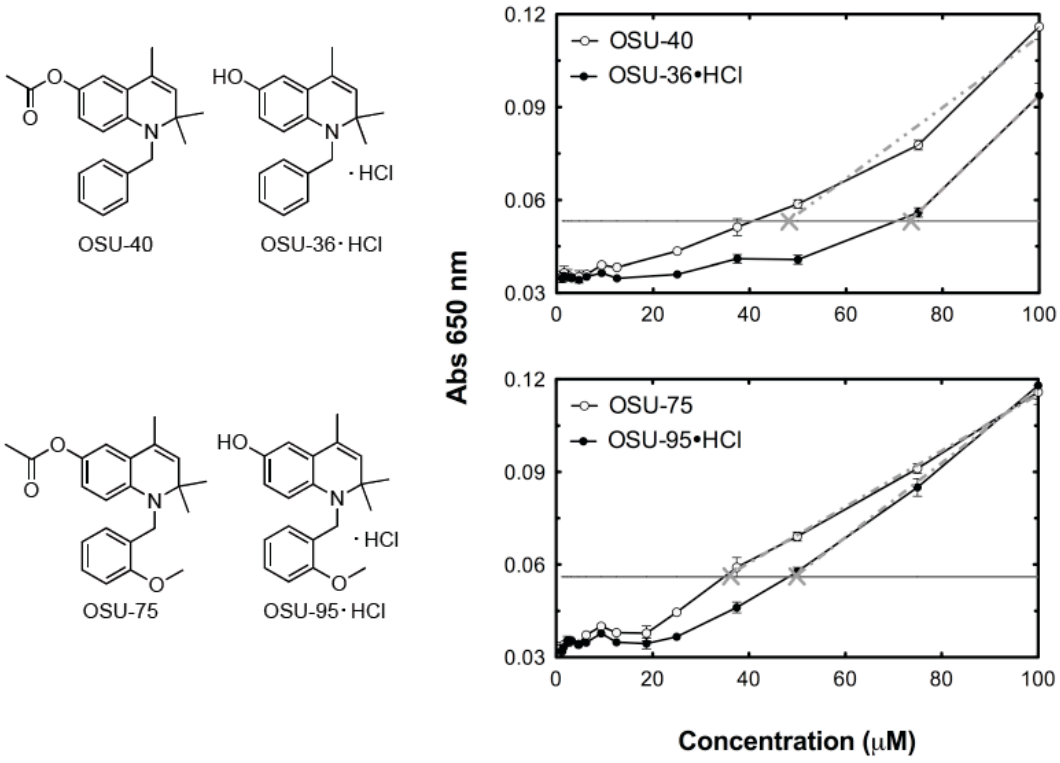
3.3 RESULTS AND DISCUSSION

3.3.1 Aqueous solubility of OSU-40, OSU-36•HCl, OSU-75 and OSU-95•HCl

The kinetic solubility assay used in this study is modified from an assay developed by Dr. Robert Jacobs and his group at Scynexis Inc. The assay and corresponding calculation were validated by comparing the results to the results from the shake-flask method carried out at Scynexis Inc. An absorbance 1.5 times greater than that of the blank was taken to indicate that the compound of interest precipitated from solution, leading to a linear increase of absorbance at 650 nm. The aqueous solubility of control compounds, including estrone, progesterone and chlorpromazine•HCl, were determined to validate the assay. The results from these assays confirmed good assay stability and the same order of solubility compared to the results obtained at Scynexis Inc.

The aqueous solubility of OSU-40, OSU-36•HCl, OSU-75 and OSU-95•HCl was determined as shown in Figure 3.1. The hydrochoride salt forms of dihydroquinolin-6-ols (OSU-36•HCl and OSU-95•HCl) showed better aqueous solubility compared to the methyl esters of their free bases (OSU-40 and OSU-75), respectively. When compared with results reported in a similar kinetic solubility assay in 1% DMSO aqueous media with slightly different assay conditions (pH = 6.5, incubation time = 10 min), the solubility of the four dihydroquinolines (35 μM -73 μM) is similar to that of several marketed drugs, such as simvastatin (31.8 μM), loratadine (32.2 μM), reserpine (59.8 μM), tamoxifen (67.3 μM) and efavirenz (76.6 μM) (119), suggesting a reasonable aqueous solubility profile of these dihydroquinolines. Testing the drugs in our assay system is needed to confirm the comparable solubility.

Carried out in 96-well plates, the advantages of this assay are fast and stable performance and the possibility of adaptation to high-throughput format. Our assay described in this chapter provides the opportunity to rank large numbers of compounds according to their aqueous solubility. However, an equilibrium solubility measurement is needed to provide precise solubility values for later pharmaceutical development of lead compounds.



Compounds	Aqueous solubility in 1% DMSO (μM)
OSU-40	48
OSU-36•HCl	73
OSU-75	35
OSU-95•HCl	50

Figure 3.2 Aqueous solubility of OSU-40, OSU-36•HCl, OSU-75 and OSU-95•HCl.

Upper left, chemical structures of OSU-40, OSU-36•HCl, OSU-75 and OSU-95•HCl. Upper right, aqueous solubility curves with absorbance at 650 nm (light scattering) plotted vs. concentration for OSU-40, OSU-36•HCl, OSU-75 and OSU-95•HCl. The gray horizontal solid line represents the baseline determined by multiplying the mean absorbance of 1% aqueous DMSO solutions lacking compound by 1.5. The gray dashed line represents the linear regression from the data points above the base line in each case. The gray cross marks the point where the linear regression crosses the base line, giving aqueous solubility in 1% DMSO of each compound, shown in the table.

3.3.2 Cytotoxicity of OSU-40 and OSU-36• HCl

As described in chapter 1, OSU-40 and OSU-36•HCl showed promising selectivity (SI = 1710 and 850 respectively) when tested against L6 rat myoblasts. The results from the panel of liver cells (Table 3.1) agreed with the previous observation and confirmed that these representative dihydroquinolines exhibit good selectivity for African trypanosomes compared to mammalian cells, with SI values ranging from 400 to 2570. This observation also supported the argument that auto-oxidation of antitrypanosomal dihydroquinolines should not lead to cell line-specific hepatotoxicity. OSU-40 generally showed less toxicity than OSU-36•HCl to all the four cell lines. This is possibly due to the better aqueous solubility of OSU-36•HCl as observed earlier. On the other hand, similar to its mode of antitrypanosomal action, OSU-40 could be hydrolyzed in liver cells to form

OSU-36, leading to the same mode of toxicity as OSU-36•HCl. This explains the observation that toxicity caused by the two compounds in the four cell lines follows the same rank: Hep3B > PLC5 > Huh7 > HepG2. The requirement for ester hydrolysis may also reduce the cytotoxicity of OSU-40 compared to OSU-36•HCl.

Compounds	**IC_{50} (μM)**				
	T. b. rhodesiense	**HepG2**	**Hep3B**	**Huh7**	**PLC5**
OSU-40	0.014	36 ± 13	8.0 ± 1.9	25 ± 6	18 ± 4
OSU-36•HCl	0.013	20 ± 4	5.2 ± 2.0	16 ± 6	11 ± 3

Table 3.1 Cytotoxicity of OSU-40 and OSU-36•HCl against liver cell lines.

3.3.3 Susceptibility of OSU-40-resistant *T. brucei*

OSU-40-resistant *T. brucei* parasites were selected through culturing the wild type parasites in the presence of increasing concentrations of OSU-40. Starting at a sublethal concentration of 0.02 μM OSU-40, the concentration of OSU-40 was increased once the parasites were adapted and showed a similar growth rate to the wild type organisms. The development of resistance was monitored by susceptibility assays (results shown in Table

3.2 and Figure 3.2). Resistance developed relatively slowly in vitro. After 14 months, OSU-40 resistant parasites showed 5.8-fold decreased susceptibility to OSU-40. Similar resistance was observed with OSU-36•HCl (4.7-fold), suggesting the decreased susceptibility was not caused by mutations in trypanosomal esterases that catalyze the conversion of OSU-40 to OSU-36. To determine whether the OSU-40-resistant cells were cross-resistant to nifurtimox and/or H_2O_2, the sensitivity of these parasites to these two compounds was also evaluated. After 14 months of exposure, there was no significant difference between the susceptibility of the OSU-40 resistant cells and the parental cells to nifurtimox or to H_2O_2.

The OSU-40 resistant parasites were maintained for a total of 22 months. Two clonal lines were developed by limiting dilution in the absence of OSU-40. Since the two clonal lines showed almost identical susceptibilities to OSU-40, OSU-36•HCl, nifurtimox, H_2O_2 and suramin, one of them was randomly selected for further study (results using the selected line are shown in Table 3.2 and Figure 3.2). As expected, clonal OSU-40 resistant cells were 21-fold less sensitive to OSU-40 and OSU-36•HCl than the parental line. Surprisingly, these parasites were 5-fold more sensitive to nifurtimox and 2.5-fold more sensitive to H_2O_2 as well. The reason for this observation is unknown. Whole genome sequencing is proposed to determine the genomic changes that led to the resistance to dihydroquinolines and hypersensitivity to nifurmox and H_2O_2.

Compound	Parental	OSU-40 resistant (14 months)		OSU-40 resistant (22 months)	
	IC_{50} (μM)	IC_{50} (μM)	Fold difference	IC_{50} (μM)	Fold difference
OSU-40	0.060	0.35	**5.8**	1.3	**21**
OSU-36•HCl	0.048	0.22	**4.7**	1.0	**21**
Nifurtimox	9.1	11	**1.2**	1.8	**0.20**
H_2O_2	330	560	**1.7**	140	**0.43**
Suramin	0.11	0.14	**1.3**	0.11	**1.0**

Table 3.2 Susceptibility of OSU-40-resistant *T. brucei* after 14 months and 22 months of OSU-40 pressure.

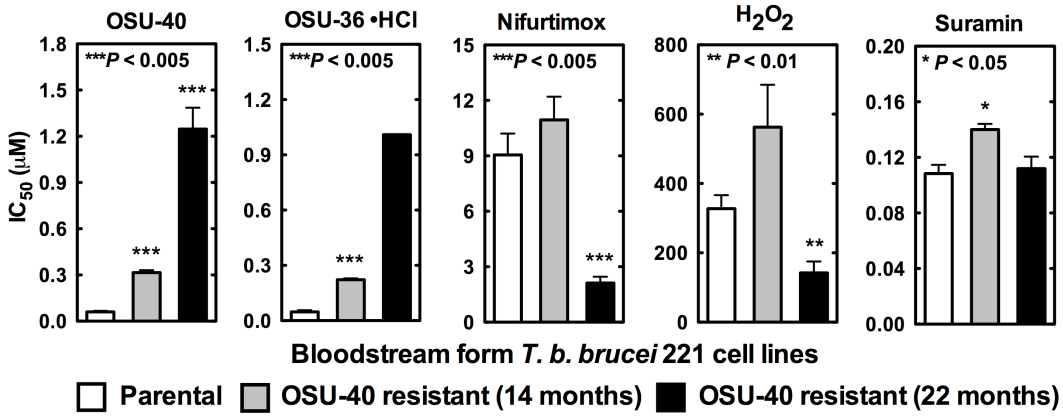


Figure 3.3 Susceptibility of OSU-40-resistant *T. b. brucei* 221 parasites to OSU-40, OSU-36•HCl, nifurtimox, H_2O_2, and suramin after 14 months and 22 months of OSU-40 pressure.

Parasites were cultured in the absence of OSU-40 pressure for 3 - 12 days prior to initiation of the susceptibility assay (see Materials and Methods). The results shown are the means of at least three independent experiments ± SE except for OSU-36•HCl at 22

months (n=1). Asterisks ((*$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$) indicate statistically significant differences in IC_{50} values from the OSU-40-resistant line to the parental line as assessed by Student's *t* test.

3.4 SUMMARY AND FUTURE DIRECTIONS

The drug-like properties of representative dihydroquinolines were determined, aiming to discover a new drug against HAT from this series of compounds. The kinetic solubility assay confirmed that the salt forms (OSU-36•HCl and OSU-95•HCl) showed improved solubility compared to the esters (OSU-40 and OSU-75), respectively. Overall, the four dihydroquinolines showed a good aqueous solubility profile when compared with that of a series of drugs. To confirm the comparable solubility, the corresponding drugs need to be tested in our assay system.

The liver cell cytotoxicity assay showed that dihydroquinolines were highly selective for trypanosomes over human liver cells. The results were also consistent with the cytotoxicity of these compounds against L6 rat myoblasts, suggesting the compounds were not more toxic to liver cells than another mammalian cell type. Both the kinetic solubility assay and liver cytotoxicity assay are fast and cost-effective, permitting these assays to be performed as routine determinations for all the new analogues in this series.

Clonal OSU-40 resistant *T. brucei* parasites were developed through 22-month selection under increasing OSU-40 pressure. These clonal cells showed dramatically lowered

sensitivity to OSU-40 and OSU-36•HCl and, to our surprise, higher sensitivity to both nifurtimox and H_2O_2. We propose to conduct whole genome sequencing to determine differences between the parental line and resistant line. Genome sequencing of drug-resistant organism has been applied to establish the mechanism of action of the drug of interest (51). The following studies will be conducted to determine the essentiality of the mutations in the resistance to OSU-40 and hypersensitivity to nifurtimox and H_2O_2.

Certain mutations, such as deletions or point mutations, can lead to lower levels of certain genes in the OSU-40-resistant line. Quantitative real-time PCR and Western blotting will be performed to evaluate the corresponding mRNA and protein levels of the gene of interest to confirm the effects of the mutation. Inducible RNA interference will be performed on wild type *T. b. b.* 221 parasites targeting the gene of interest. The susceptibility of the RNAi lines to dihydroquinolines, nifurtimox and H_2O_2 will be determined using the approaches described in Chapter 2. The induced RNAi line exhibiting downregulation of the targeted gene should show the same phenotypic characteristics, such as resistance to dihydroquinolines and/or hypersensitivity to nifurtimox and H_2O_2, if the mutation is responsibile for altered susceptibility in the OSU-40 resistant parasites.

On the other hand, if gene duplication is detected in the OSU-40-resistant line, quantitative real-time PCR and Western blotting will be conducted to confirm the increased level of corresponding mRNA and protein. Inducible RNA interference will be performed on OSU-40-resistant parasites targeting the gene of interest. The susceptibility

of the RNAi lines to dihydroquinolines, nifurtimox and H_2O_2 will be determined using the approaches described in Chapter 2 as well. The induced RNAi line displaying downregulation of the targeted gene should show reversed phenotype characteristics, such as increased sensitivity to dihydroquinolines and/or decreased sensitivity to nifurtimox and H_2O_2, if the mutation is the essential reason for the corresponding effect in the OSU-40 resistant parasites.

These studies will shed light on the mechanism of resistance to dihydroquinolines and possibly provide more evidence concerning the mode of action of these compounds. This may also provide clues regarding the origin of the heightened sensitivity of the parasites to nifurtimox. Such information could be of great importance to new antitrypanosomal drug discovery efforts.

CHAPTER 4: CANINE LEISHMANIASIS

Leishmaniasis is a parasitic disease caused by more than 20 species of the protozoan *Leishmania*. The disease threatens up to 350 million people in 88 countries around the world (64, 134). According to WHO, 12 million individuals are estimated to be infected with about 1 - 2 million estimated new cases occurring every year (134). The protozoan is transmitted by the bite of phlebotomine sand flies. Depending on the species of *Leishmania* and the host's immune system, the disease can lead to a wide range of clinical symptoms. These symptoms are commonly characterized into three categories: cutaneous, mucocutaneous or visceral (13, 30, 64). Visceral leishmaniasis (VL) is the most severe form and leads to most of the 70,000 deaths each year caused by leishmaniasis (64).

Depending on the geographical distribution and causative *Leishmania* species, VL can be anthroponotic, i.e., transmitted only from human to vector to human. Zoonotic VL occurs when the parasites are transmitted from animal to vector to human; a representative life cycle is shown in Figure 4.1, A, B and C (2). *Leishmania infantum* (*L. i.*) is the most widespread species causing zoonotic VL with domestic dogs as the main reservoir. Cats (73), wild canids (115) and horses (37) are also reported as reservoirs where canine

leishmaniasis distributes. Canine leishmaniaisis caused by *Leishmania infantum* is one of the major zoonotic diseases that lead to death in dogs. As a common companion animal, the health of dogs is of great concern in pet owners in developed and developing countries.

4.1 DISTRIBUTION

Canine leishmaniasis due to *Leishmania infantum* exists in about 50 countries in Europe, Africa, Asia and the Americas (117) (shown in Figure 4.1). Although the majority of human VL cases occur in Bangladesh, Ethiopia, India, Nepal, Sudan and Brazil (18, 30), canine leishmaniasis has been mostly identified in the Mediterranean basin, the Middle East, Central Asia, and China (2, 72). Sporadic cases have been reported in canine travelers returning to the United States from endemic areas (93, 105). In 2000, Foxhounds were reported to be infected with *Leishmania infantum* by a kennel in New York State (41, 93). By 2005, seropositive Foxhounds had been reported in 60 kennels in 22 states and 2 Canadian provinces (93, 101).

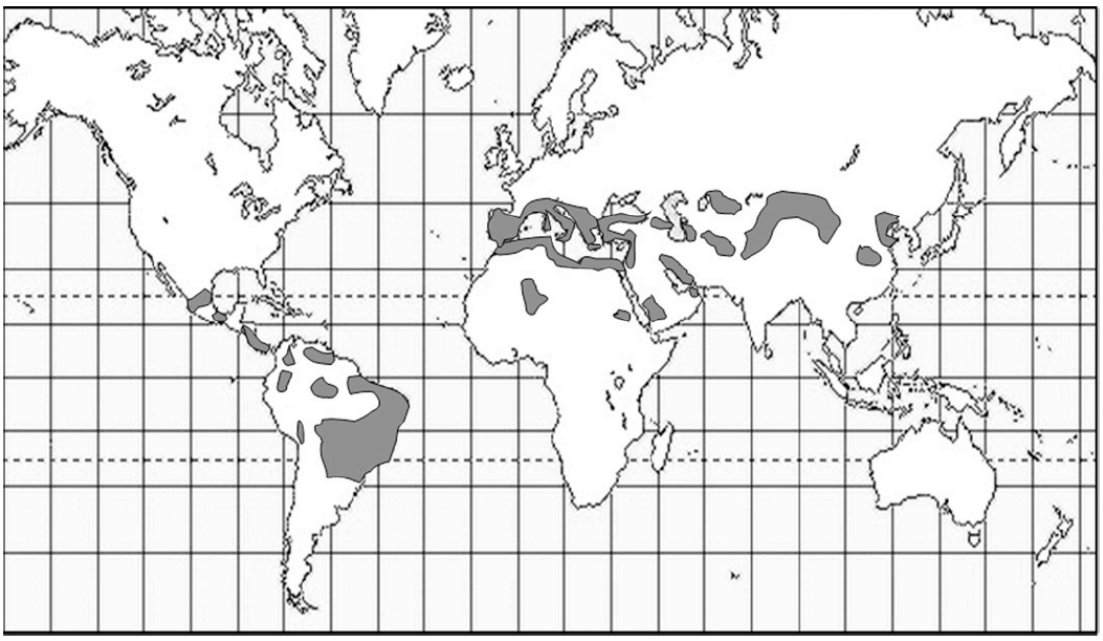


Figure 4.1 Distribution of zoonotic visceral leishmaniasis caused by *Leishmania infantum.*

[Reprint from Gramiccia M (44) with permission from Elsevier]

4.2 Parasite Transmission and Life cycle

Different subspecies of *Leishmania* share similar life cycles, in which parasites exist in two forms – promastigotes and amastigotes (13). Promastigotes are elongated, with an anterior flagellum originating from a kinetoplast while amastigotes are round to oval shaped and only retain a rudimentary flagellum. The life cycle of *Leishmania infantum* is summarized below with the numbered steps shown in Figure 4.2 (76):

① *Leishmania infantum* promastigotes are injected by female phlebotomine sand flies while taking a blood meal on humans or dogs and then phagocytized by macrophages and/or other types of mononuclear phagocytic cells.

② - ⑦ Promastigotes transform into amastigotes within these host cells. Amastigotes actively replicate in phagocytes, leading to cell destruction, and progressively infect more phagocytes. Once the organisms have entered the systemic circulation, they can again be taken in during blood meals by sand flies.

⑧ - ⑩ Within sand flies, amastigotes transform into promastigotes in the gut and migrate to the proboscis. Metacyclic promastigote forms found in the proboscis are infective for mammalian hosts, permitting the life cycle of the parasite to continue.

Interestingly, although both life stages of the parasite are capable of replication via binary fission, promastigotes only multiply in the sand fly and will lose the ability to replicate once injected into the host bloodstream; amastigotes only replicate in phagocytic cells.

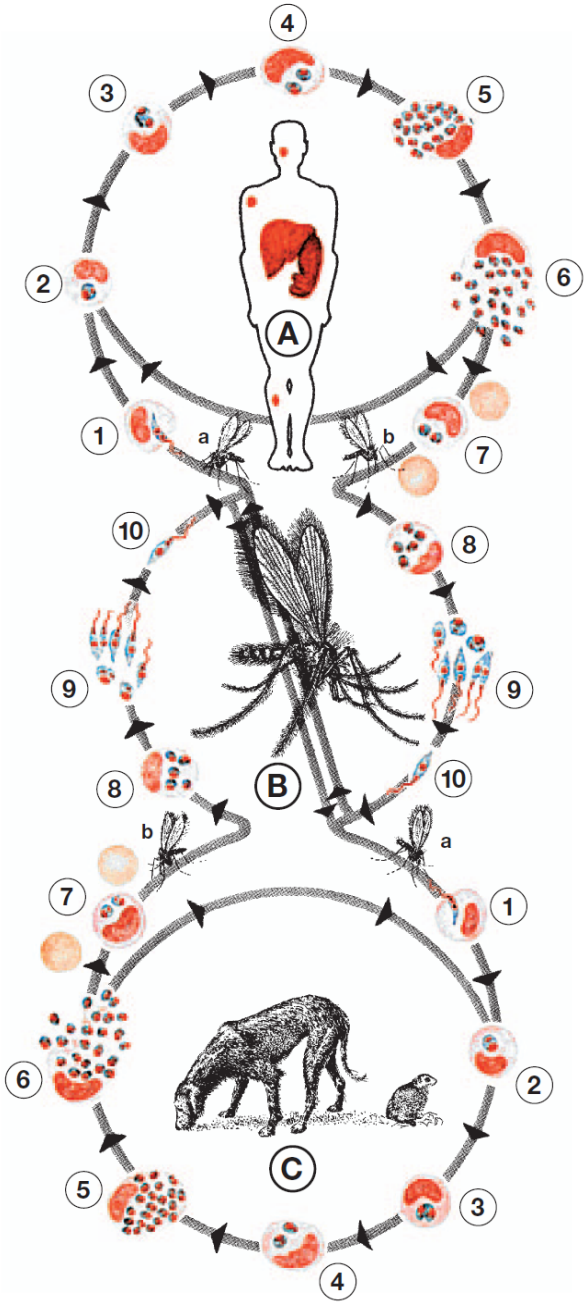


Figure 4.2 Life cycle of *Leishmania infantum.*

[Springer and the Parasitology Research, 90, 2003, S108-S111, Repellent efficacy of a combination containing imidacloprid and permethrin against sand flies (Phlebotomus papatasi) on dogs, Mencke, N., P. Volf, V. Volfova, and D. Stanneck, Figure 1, is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media (76)]

4.3 CLINICAL MANIFESTATIONS

Canine leishmaniasis leads to variety of clinical features, and skin lesions on the head are the most frequently reported manifestation. The incubation period also differs from case to case, lasting from weeks to years (2, 76). The disease can be asymptomatic in its early stage and cutaneous symptoms can possibly be observed after months of infection (2). Cutaneous symptoms start as a single papule, which enlarges into a nodule or lesion with edges (93). Although the parasites normally accumulate in bone marrow, liver and spleen in human VL cases (64), they distribute extensively in the organs of infected dogs, including the skin, spleen, liver, lymph glands bone marrow and kidney etc. (2). A study involving 93 infected dogs by Semiao-Santos et al. (108) showed that lymphadenopathy, onychogryphosis and cutaneous lesions occurred in more than 50% of the cases. The presence of *Leishmania* parasites in different tissues and organs leads to ulcers and bald patches after months to years of infection and death can occur, usually due to renal or hepatic failure (2).

4.4 DIAGNOSIS

Direct methods	Indirect methods
• Cytologic evaluation: microscopic observation of *Leishmania* amastigotes extracellarly or in macrophages.	• Polymerase chain reaction (PCR) assay: amplification of *Leishmania* specific gene fragments for detection.
• Histological evaluation: detection of parasites in tissue sections from lesions.	• Serological methods:
• Organism culture	Indirect fluorescence antibody (IFA)
• Xenodiagnosis: examination of phlebotomine vectors that fed on a dog with suscepted infection.	Enzyme-linked immunosorbent assay (ELISA)
	Western blotting
	Immunochromatographic tests
	• Evaluation of cellular immune response

Table 4.1 Diagnostic methods for canine leishmaniasis (2, 18, 88).

The diagnostic methods used in dogs (88) are similar to those employed in humans (18). Multiple factors, such as history, clinical findings, and results from basic biochemical laboratory tests should be taken into consideration prior to diagnosis. Diagnosis is made based on the observation of parasites through direct methods and/or indirect methods as shown in Table 4.1.

4.5 TREATMENT AND PREVENTION

Treatment of canine leishmaniasis is carried out with the same drugs used in humans. The most common antileishmanial drugs used in dogs (chemical structures shown in Figure 4.3) are listed in Table 4.2 in descending order of citations, together with the proposed modes of action and limitations (2, 18, 82). As an example of the application of these treatments, Meglumine antimonite (GLUCANTIME) will be further discussed in detail. This drug is used to treat leishmaniasis in both humans and dogs. It is reported with a good clinical efficacy and reduces clinical signs usually in 1 or more weeks. Improved biological values are also observed with histological and/or serological detection methods. However, the treatment of meglumine antimonite failed to fully eliminate Leishmania parasites in multiple studies (81, 82), as parasites were detected in tissues of dogs a few months after treatment. Clinical relapses can occur months to 1-2 years after treatment. The most common adverse effects are pain and swelling of the injection site, and fever, diarrhea, loss of appetite have also been reported (114). Meglumine antimonite

can be used in combination with allopurinol and this combination therapy is the most common treatment of canine leishmaniasis. A longer period of clinical remission can be achieved with combination therapy compared to monotherapy with each drug. However, the combination therapy is still not able to eliminate the parasites in infected dogs (29).

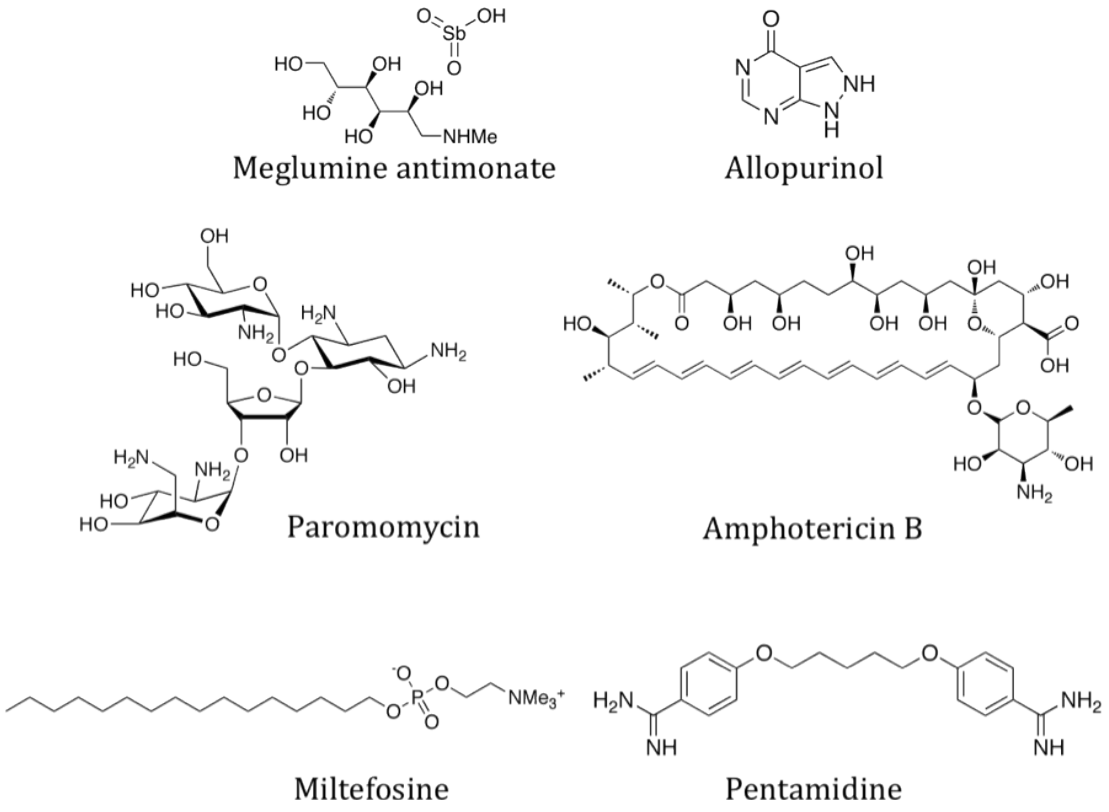


Figure 4.3 Chemical structures of the drugs used in canine leishmaniasis chemotherapy.

In terms of prevention, the likelihood of canine visceral leishmaniasis can be reduced by treating dogs dermally with synthetic pyrethroids, which are lethal for the sand fly if the vector lands on treated dogs for a sufficient time to absorb a lethal dose (72). If there is only brief contact between the flies and insecticide-treated skin, pyrethroids could also lead to irritation and disorientation in the vector, consequently reducing the blood-feeding rate.

As listed in Table 4.2, drugs currently on the market for the treatment of human leishmaniasis show highly toxic effects and/or parasite resistance. In addition, all known antileishmanial drugs used in dogs only lead to the temporary or permanent remission of clinical signs; none of them eliminate the infection (3) and clinical relapses commonly occur after treatment (2). Also, since all the treatments were discovered and developed through human clinical studies and then adapted to dogs, the pharmacokinetic profile of the drug may not be optimal for the treatment of canines. While preventive treatments with pyrethroids is effective in reducing the incidence of sand fly bites, non-sand fly transmission routes are suspected to be a problem. In the outbreak of canine leishmaniasis in a kennel in Duchess County (New York State, USA) in 2000 (41, 93), infected sand flies were not found. Transmission has also been reported through blood transfusion in dogs (2). Considering the factors outlined above, new agents specifically targeting canine leishmaniasis are needed.

Drug	Mode of action (**Not fully elucidated)	Limitations
Meglumine antimonate	Affects energy metabolism, e.g. leishmanial glycolysis and fatty acid oxidation **	• Occurrence of resistance in certain regimens
Allopurinol	Incorporates into RNA synthesis and inhibits protein synthesis.	• Moderate efficacy • In combination therapy, usage does not improve the initial response to treatment, only delays the onset of relapse and reduces frequency.
Paromomycin	Acts on ribosomes and blocks protein synthesis **	• High incidence of adverse effects
Amphotericin B	Interacts with ergosterol in the parasite membrane, leading to membrane structural disorganization.	• Severe adverse effects • Complexity of formulation in preparation and administration • Usage discouraged by WHO to avoid occurrence of resistant *Leishmania* strains
Miltefosine	Affects cell membrane synthesis	• Limitations not well documented
Pentamidine	Affects mitochondria, e.g. mitochondrial membrane potential and kinetoplast DNA synthesis **	• Severe adverse effects

Table 4.2 Current drugs for the treatment of canine leishmaniasis

CHAPTER 5: DEVELOPMENT OF HIGH THROUGHPUT ANTILEISHMANIAL SCREENING ASSAY

As discussed in detail in Chapter 4, drugs applied in therapy for canine leishmaniasis are not satisfactory due to the following reasons: 1) None of them can eliminate the infection permanently without relapse; 2) Adapted from therapy for human VL, preclinical characterizations are not relevant to dogs; and 3) There are concerns of selecting drug-resistant *Leishmania* strains when using certain drugs in the treatment of dogs with leishmaniasis. Studies on new combination therapy (77) and canine vaccines (25, 80, 104) have been reported targeting canine leishmaniasis, but the discovery and development of novel drugs against this disease caused by *Leishmania infantum* has not been reported.

5.1 AIMS OF STUDY

The ultimate goal of this project is to discover novel candidates as preventive reagents against canine leishmaniasis. To achieve prophylactic activity, the candidates must show

a fast-killing effect on *Leishmania infantum* promastigotes. High throughput technology provides a potentially low-cost and rapid approach to facilitate antileishmanial lead discovery, particularly when the target is the promastigote form of the parasite due to its ease of culture. Although multiple antileishmanial high throughput screening efforts have been reported targeting human leishmaniasis using *L. donovani* and *L. major* as target species (27, 85, 113), high throughput screening against *Leishmania infantum* has not been reported. In this project, a high throughput assay is developed and validated on the promastigote form of *Leishmania infantum*. A similar screening assay has also been developed using *Leishmania donovani* promastigotes to address the question of whether screening against *Leishmania donovani* can provide accurate hits against canine leishmaniasis caused by *Leishmania infantum*.

5.2 MATERIALS AND METHODS

All chemicals were purchased from Aldrich Chemical Co. unless stated otherwise. A library of 427 compounds was available in house (Pfizer Animal Health, VMRD, Kalamazoo, MI).

5.2.1 Validation of antileishmanial screening assay

5.1.1.1 Parasite culture

L. infantum strain (gift from Dr. Hechmi Louzir, Laboratoire d'immunologie, Institut Pasteur de Tunis, Tunisia) was isolated from a dog lymph node aspirate, and then transformed from amastigote forms into promastigotes. *L. infantum* and *L. donovani* S1 (Sudanese strain) promastigotes were cultured in a shaking incubator at 25°C in RPMI-1664 medium containing L-glutamine, streptomycin and penicillin G supplemented with 20% and 10% fetal bovine serum (FBS) (J R Scientific Inc., Woodland, CA) respectively.

5.1.1.2 Validation of the endpoint indicator

To access the efficiency and accuracy of the CellTiter-Glo luminescent cell viability assay kit (CellTiter-Glo, Promega, Madison, WI) as the endpoint indicator, the relationship between relative luminescence units (RLU) and *Leishmania* parasite number in each well was evaluated. Serial 2-fold dilution of parasites from an exponential phase culture (1-5 $\times$ 10^7 parasites/ml) was carried out in sterile 384-well white opaque microplates (PerkinElmer, Waltham, MA), followed by the addition of CellTiter-Glo into each well. The presence of metabolically active cells was signaled by the quantitation of ATP present and recorded with a luminescence spectrometer (PerkinElmer, Waltham, MA).

5.1.1.3 Validation of assay conditions

Reference compounds, including pentamidine, amphotericin B and miltefosine, were serially diluted in DMSO using Bravo Automated Liquid Handling Platform (Agilent Technologies, Santa Clara, CA) and dispensed using Mosquito® Accurate Nanolitre to Microlitre Pipetting System (TPP LabTech Inc., Cambridge, MA) into assay microplates in a volume of 0.24 µl/well. Exponentially growing *Leishmania* parasites were diluted in culture medium and seeded to give 1×10^5 parasites/40 µl in each well using a multidrop combi reagent dispenser (Thermo Fisher Scientific, Waltham, MA). Plates were then incubated in a shaking incubator at 25 °C for 24 hrs before the addition of 20 µl of CellTiter-Glo® into each well. The antiproliferative effect was assessed by measuring the luminescence from the remaining metabolically active cells in each well and recorded with a luminescence spectrometer. IC_{50} values were determined using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Pentamidine was used as the positive control with a final concentration of 20 µM, and 0.24 µl DMSO (0.6% v/v) was used as negative control. Assay validation was performed in 2 independent experiments with 12 plates, including 4 plates with 0.6% DMSO in every well, 4 plates with positive and negative controls in wells with a checkerboard pattern, and 4 plates with serial dilutions of reference compounds. The plate settings are shown in Figure 1. Z' was determined for HTS assay validation with the following formula.

$$Z' = 1-(3\sigma_p+3\sigma_n)/(|\mu_p-\mu_n|)$$

σ_p, μ_p, σ_n and μ_n are the standard deviation and mean values of positive (p) control and negative (n) controls, respectively.

5.2.2 Screening against *Leishmania* parasites

A library of 427 compounds was dissolved in DMSO and screened at 10 μM in 0.6% DMSO using the conditions validated above against *L. infantum* and *L. donovani* promastigotes. The following formula was used to calculate the percentage inhibition of parasite growth for each compound:

$$\% \text{ Inhibition} = 100-(RLU_x-RLU_p)/(RLU_n-RLU_p)\times 100$$

RLU_x, RLU_p and RLU_n were the RLU when treated with the compound (*x*), positive control (*p*) and negative control (*n*), respectively. Compounds showing more than 40% inhibition were identified as hits and the available ones were further evaluated with dose response curves using the same procedure described for reference compounds. The Z' factor was evaluated for each plate in the screening.

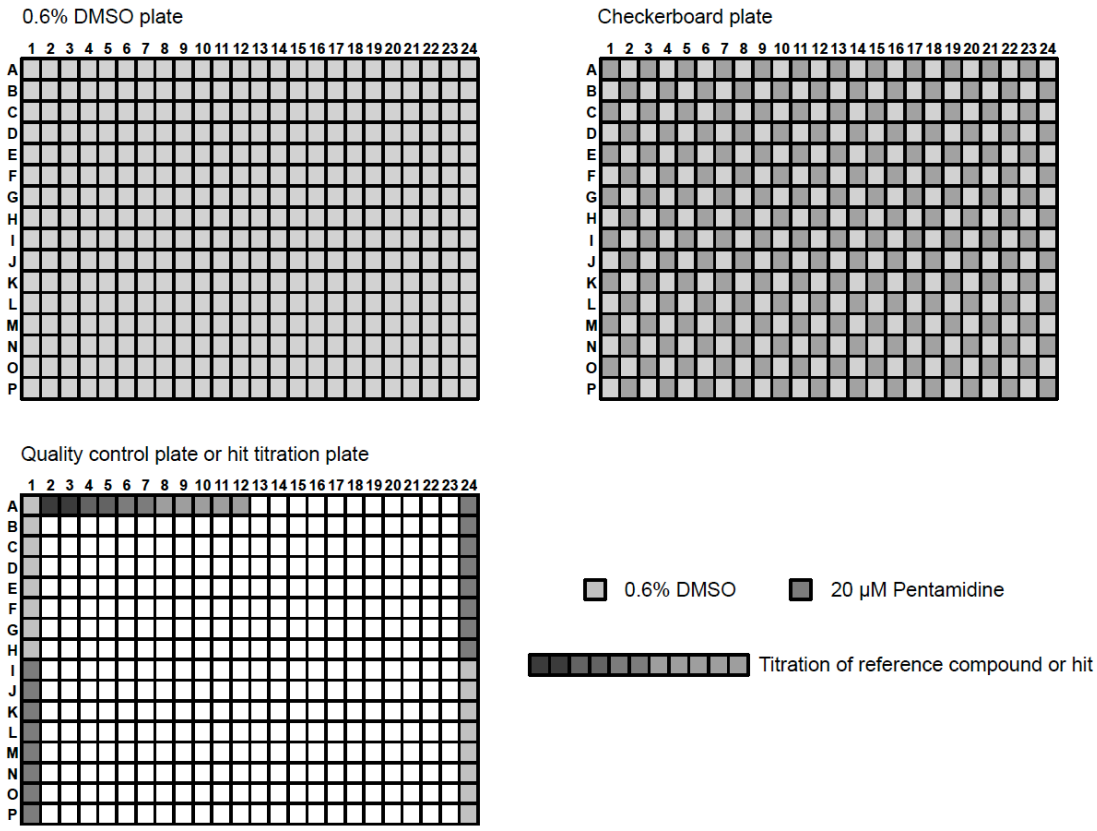


Figure 5.1 Plate setting for assay validation and hit titration.

Each type of plates contains: Upper left, 0.6% DMSO in every well; Upper right, positive (20 µM of pentamidine) and negative (0.6% DMSO) controls in wells with a checkerboard pattern; Lower left, wells A1-H1 and I24-P24 contain 0.6% DMSO, wells I1-P1 and A24-H24 contain 20 µM pentamidine, other wells contain horizontal serial dilutions of reference compounds, pentamidine, amphotericin B and miltefosine, with different starting concentration in quality control plates or serial dilutions of hits starting at 120 µM in hit titration plates.

5.3 RESULTS AND DISCUSSION

The ultimate goal of this screening effort is to discover preventive agents that will be administered before infection, persist in the bloodstream at a certain concentration and most importantly have the ability to kill the *Leishmania* parasites if they are injected by the vector. In this case, the organism we are targeting is the promastigote form of the parasite prior to phagocytosis and subsequent transformation into amastigotes.

5.3.1 Luminescence output correlates with parasite number per well

CellTiter-Glo determines the number of metabolically active parasites based on the quantitation of ATP. To assess the applicability of CellTiter-Glo as the endpoint indicator for *L. infantum* and *L. donovani* viability in high throughput assays, the relationship between RLU and *Leishmania* cell density in each well was evaluated. For both *L. infantum* and *L. donovani*, a strong linear relationship was observed between parasite number and RLU (Fig 5.2A). Resazurin (113, 125), MTT followed with lysis buffer (83), and MTS/PMS (40) have been reported as end point indicators in antileishmanial screening assays. Compared to these reagents, CellTiter-Glo provides the advantage of sufficient lysis and instant signaling without addition of lysis reagents or further incubation.

5.3.2 Dose-response curves of reference compounds

To validate the conditions for high throughput screening on the promastigote form of both *L. infantum* and *L. donovani* in a 384-well format, the dose-reponse curves and IC_{50} values of reference compounds were determined (Figure 5.2B). IC_{50} values of reference compounds were within the range of 4 × higher or lower than multiple reported values (40, 85, 113, 125), suggesting good reliability of the assay conditions. Interestingly, miltefosine showed significantly different activity on *L. donovani* and *L. infantum*, suggesting the possibility of identifying reagents with selectivity for one species of *Leishmania* compared to another.

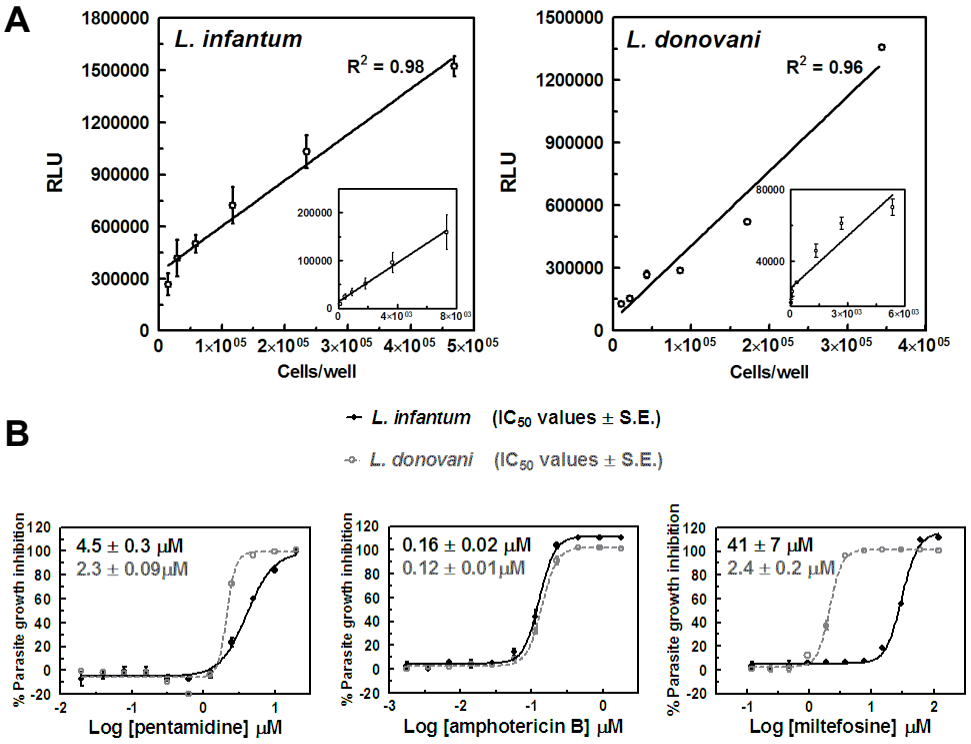


Figure 5.2 Assay validation.

A) Luminescence output correlates with cell density for *L. infantum* and *L. donovani.* A linear relationship exists between luminescence measured with the CellTiter-Glo and the number of cells (R^2 = 0.98 and 0.96 for *L. i.* and *L. d.,* respectively). Serial 2-fold dilution of *Leishmania* parasites was made in 25 µl of medium in 384-well microplates. Luminescence was recorded 20 minutes after addition of 25 µl of CellTiter-Glo reagent in each well using a PerkinElmer luminescence spectrometer. Values represent the mean ± S.E. of 3 replicates for each cell density. B) Dose response curves for pentamidine, amphotericin B and miltefosine. The black line represents results from *L. infantum* and

the gray dashed line represents results from *L. donovani*. IC_{50} values are the means of results from at least three independent experiments; representative curves are shown in the figure.

5.3.3 High throughput screening

A library of 427 compounds was screened at 10 μM against both *L. infantum* and *L. donovani*. 0.6% of DMSO was included in the individual assay wells to allow a concentration of 10 μM compound in the initial screening. To select hits producing rapid killing of the parasites, a duration of 24 hrs was selected for the screen. For the screen on *L. infantum* and *L. donovani*, the average Z' values calculated from positive and negative controls in each plate was 0.72 and 0.86, respectively (Figure 5.3). As Z' > 0.5 has been the widely accepted criterion for a good robustness in high throughput screening assays, the Z' values from both of screens on *L. infantum* and *L. donovani* suggested that the assay conditions developed gave a satisfactory robustness. From these plates, average values for the percent growth inhibition from three separated experiments were obtained. At least 40% inhibition was chosen as the cut-off criterion to select the most active compounds as hits.

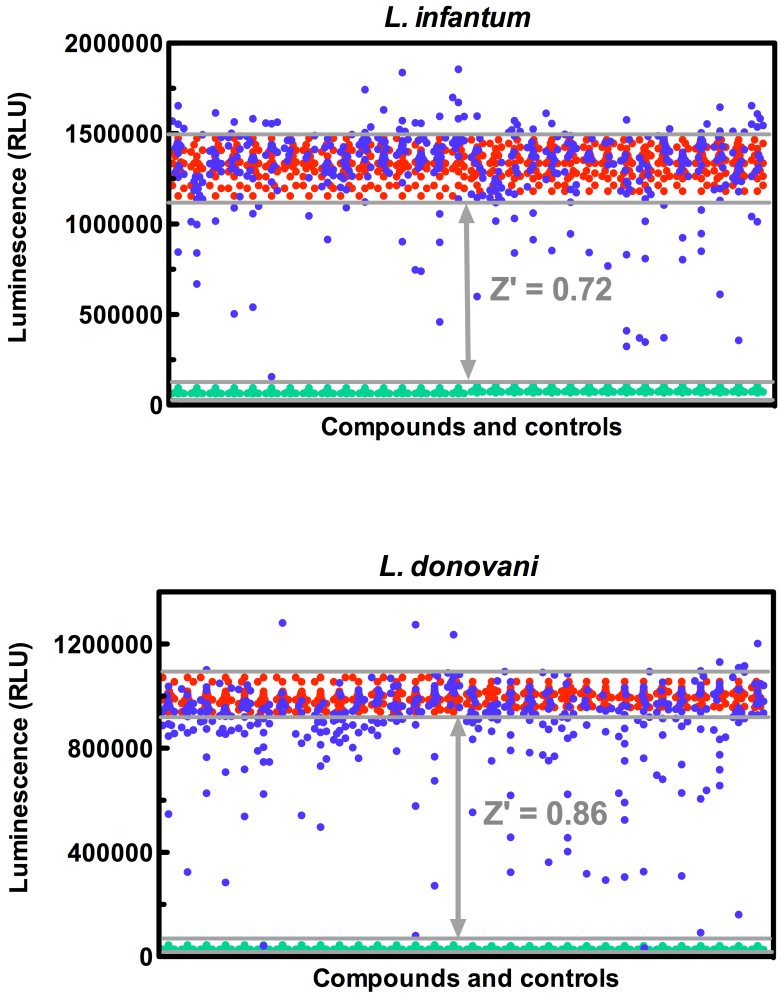


Figure 5.3 Distribution plot of RLU values from assays on *L. infantum* and *L. donovani* promastigotes.

Blue dots, 427 compounds at 10 μM; Red dots, 0.6% DMSO; Green dots, pentamidine at 20 μM.

In the *L. infantum* screening effort, Twenty-one compounds (4.9%) showed growth inhibition above or equal to 40% and were selected for further investigation. From *L. donovani* plates, 27 compounds (6.3%) showed growth inhibition above or equal to 40%, among which 16 were also active on *L. infantum* (Figure 5.4). A total number of 24 hits were available for further determination of IC_{50} values on the two parasite species.

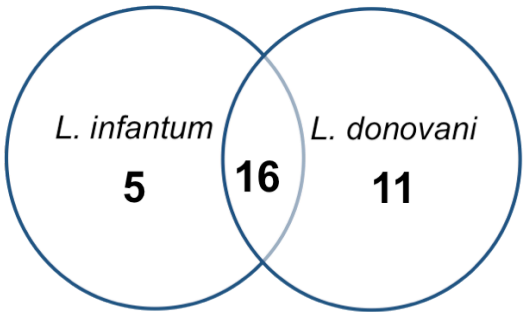


Figure 5.4 Number of hits on *L. infantum* and/or *L. donovani*.

From this initial screening with 40% inhibition as the cut-off criterion, the hits for *L. infantum* and *L. donovani* did not overlap completely. Assuming that *L. donovani* was utilized as a representative species to screen for activity against *L. infantum* with the same conditions developed in this study, inhibition of *L. donovani* will give 16 hits that should be selected against *L. infantum*; 5 of the hits on *L. infantum* (24%) would not be selected. On the other hand, from *L. donovani* screening, 11 hits among the total number of 27 showed inhibition < 40% against *L. infantum*, giving a false positive rate of 11/27 ×

100% = 40 %. Overall, under these conditions, *L. donovani* is not an ideal alternative species to screen for compounds that are active on *L. infantum*.

5.3.4 Antileishmanial activity of hits

IC_{50} values of the 24 hits were determined for both *L. infantum* and *L. donovani*, and the results are listed in Table 5.1. Eighteen of these molecules (75%) displayed IC_{50} values < 25 μM against *L. infantum*, *L. donovani*, or both parasites, suggesting good selectivity in the initial screening.

Comparing the IC_{50} values of all 24 hits against *L. infantum* to the results against *L. donovani*, eighteen of the compounds showed IC_{50} values within a 2-fold difference. Among the 6 exceptions, compounds 19, 20 and 21 showed over 2-fold higher activity on *L. donovani*; compounds 7 and 16 were not active against *L. infantum* at 120 μM, but showed moderate activity on *L. donovani*. Overall, five of the 24 hits (21%) in this study showed more than 2-fold higher activity on *L. donovani*, consistent with our conclusion from initial screening that screening on *L. donovani* to identify hits against *L. infantum* can give a high false positive rate. On the other hand, compound 18 was 2.1-fold more active on *L. infantum*, suggesting the possibility to discover selective reagents on *L. infantum* over *L. donovani.*

Regarding the most active molecules, three compounds (2, 14 and 17) exhibited IC_{50} values lower than 5 μM against both *L. infantum* and *L. donovani* and three of them (19,

20 and 21) displayed IC_{50} values < 5 μM against *L. donovani* only. The activity of these potent compounds was comparable to the results obtained with the standard compound pentamidine (Table 5.4), a drug used to treat canine leishmaniasis.

Compound	**% Inhibition at 10 μM**		**IC_{50} (μM)[a]**	
	L. infantum	***L. donovani***	***L. infantum***	***L. donovani***
1	47	95	6.9 ± 1.6	5.4 ± 0.1
2	94	99	3.6 ± 0.4	4.1 ± 0.3
3	63	47	6.4 ± 0.7	6.9 ± 1.1
4	46	43	37 ± 7	39 ± 9
5	69	75	24 ± 7	28 ± 6
6	66	73	7.8 ± 1.3	7.5 ± 1.1
7	22	46	> 120	17 ± 8
8	33	51	40 ± 18	31 ± 4

[a]Values represent the mean ± S.E. of 3 replicates

Table 5.1 Antileishmanial activity of hits.

Compound	% Inhibition at 10 µM		IC_{50} (µM)[a]	
	L. infantum	*L. donovani*	*L. infantum*	*L. donovani*
9	38	46	15 ± 5	8.1 ± 1.1
10	52	69	15 ± 2	8.1 ± 0.7
11	77	33	8.5 ± 1.5	10 ± 1
12	-21	46	> 120	> 120
13	81	49	37 ± 4	39 ± 9
14	74	72	1.3 ± 0.3	1.5 ± 0.4
15	79	87	33 ± 3	17 ± 0
16	-2	42	> 120	43 ± 11
17	77	11	3.2 ± 0.5	4.2 ± 0.6
18	59	7	8.9 ± 2.3	19 ± 2
19	39	66	7.6 ± 3.9	2.3 ± 1.1

[a]Values represent the mean ± S.E. of 3 replicates

Table 5.1 Antileishmanial activity of hits. (Continued)

Compound	% Inhibition at 10 µM		IC_{50} (µM)[a]	
	L. infantum	*L. donovani*	*L. infantum*	*L. donovani*
20	40	70	13 ± 8	3.9 ± 2.0
21	39	94	8.7 ± 1.3	4.1 ± 0.2
22	25	56	23 ± 3	25 ± 11
23	41	38	31 ± 3	38 ± 4
24	31	40	8.4 ± 1.5	7.9 ± 0.3
Pentamidine	---	---	4.5 ± 0.3	2.3 ± 0.1

[a]Values represent the mean ± S.E. of 3 replicates

Table 5.1 Antileishmanial activity of hits. (Continued)

5.4 SUMMARY AND FUTURE DIRECTIONS

High throughput screening assays on *L. infantum* and *L. donovani* were designed, developed and validated in a rapid and simple 384-well format with a satisfactory robustness (Z' = 0.71 and 0.86, respectively). The major advantage of these assays are 1) All the liquid handling steps are automated, and 2) The assays are in 384-well format and

has the potential to be adapted into 1536-well format considering the culture conditions of the leishmania parasites. Although for assay validation described in this chapter, the screening was only carried out with a small library of 427 compounds, the high throughput screening assays developed have great potential to be utilized in screening of large numbers of compounds against *L. infantum* and *L. donovani*. Screening against *L. infantum* and *L. donovani* in parallel provides the opportunity to find new canine antileishmanial prophylactics.

From the screening of a 427-member library, six compounds (2, 14, 17, 19, 20 and 21) exhibited IC_{50} values that are comparable to pentamidine (lower than 5 μM) against *L. infantum* and/or *L. donovani.* Comparison of the *L. infantum* screen to the *L. donovani* screen indicates that screening against *L. donovani* is not an ideal approach to identify hits against *L. infantum* because of a high false positive rate.

Identification of structural clusters of the 24 hits is proposed to further analyze the structure-activity relationship. Hits exhibiting IC_{50} values lower than 5 μM against *L. infantum* and/or *L. donovani* will be evaluated in an intracellular amastigote assay. This may provide information on their selectivity on the promastigote form over amastigote form.

To discover prophylactic agents for canine leishmaniasis, this initial screening assay should be followed with secondary cytotoxicity screening on mammalian liver cells and evaluation of physical and chemical properties. The antileishmanial screening assay

against *L. infantum* developed in this study is a good initiation point leading to new drug candidates to the discovery pipeline for canine leishmaniasis.

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