ACCUMULATION AND INTRACELLULAR DISTRIBUTION OF AROMATIC DIAMIDINES IN AFRICAN TRYPANOSOMES

AMANDA MARIE MATHIS

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Approved by:

Dr. James E. Hall, Advisor

Dr. Gary Pollack, Committee Chair

Dr. Richard R. Tidwell, Reader

Dr. Moo Cho, Reader

Dr. David Wilson, Reader

ABSTRACT

Amanda Mathis: Accumulation and Intracellular Distribution of Aromatic Diamidines in African Trypanosomes (under the direction of James E. Hall)

Human African Trypanosomiasis (HAT), also known as sleeping sickness, is a devastating disease that affects millions of people in the poor nations of sub-Saharan Africa. There is a great need for new treatments of this disease, as only four drugs are currently available, and all are associated with toxicity and other problems. The diamidine, pentamidine, has long been used for the treatment of trypanosomiasis, despite its toxicity and lack of knowledge of its mechanism of action. Drug development efforts have recently focused on creating less toxic, more potent diamidine derivatives of pentamidine with an orally bioavailable formulation. DB289, the prodrug of DB75, is in Phase III clinical trials as an oral treatment of HAT. Almost 2000 diamidines and related compounds have been synthesized, many with potent anti-trypanosomal activity. Although DB75 and other diamidines are potent DNA binders, the overall mechanism of action of this series of compounds is unknown.

The focus of this research is to investigate diamidine accumulation and distribution in trypanosomes in vitro and in vivo. Diamidines such as DB75 accumulate to high concentrations in vitro and in vivo in trypanosomes, and localize to the kinetoplast and nucleus, which contain DNA, as well as the acidocalcisomes. The increase in treatment failures of anti-trypanosomal compounds has been a major concern in recent years, especially with melarsoprol. Several trypanosome lines have been developed which are resistant to

DB75 in the laboratory, and methods developed to investigate intracellular accumulation and distribution were extended to these lines. These lines lack the P2 transporter, thought to be the main uptake transporter for DB75. DB75 accumulation was lower in the resistant lines, with intracellular distribution also altered. Total accumulation, however, was substantial in these lines, despite lack of the major uptake transporter described for this compound.

Benzofuran and benzimidazole diamidines were also investigated. The benzofuran series was found to accumulate in non-DNA containing organelles, which may represent a new mechanism of action for these diamidines. Together, the studies in this dissertation have proven that diamidines selectively accumulate in trypanosomes and that there is likely more than one mechanism of action for diamidines.

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ABBREVIATIONS

AUC	Area under the Concentration Time Curve
CBMEM	Complete Baltz Modified Essential Medium
CBSS	Carter's Balanced Salt Solution
CNS	Central nervous system
CSF	Cerebrospinal fluid
$DB75^{R}$-CL1	DB75 resistant line of trypanosomes
DFMO	Difluoromethylornithine, or eflornithine
FBS	Fetal Bovine Serum
HAPT1	High Affinity Pentamidine Transporter 1
HAT	Human African trypanosomiasis
HPLC	High Performance Liquid Chromatography
IC_{50}	50% inhibitory concentration
IP	Intraperitoneal administration
IV	Intravenous administration
LAPT1	Low Affinity Pentamidine Transporter 1
Km	Concentration of substrate associated with half of the maximum accumulation rate
LC/MS	Liquid Chromatography/Mass Spectrometry
MSF	Médicins sans Frontières, or Doctors without Borders
ODC	Ornithine decarboxylase enzyme
P2KO	P2 Transporter knockout
PCR	Polymerase Chain Reaction
PSG	Phosphate saline glucose buffer

TbAT1	*T. brucei* Aminopurine Transporter 1, or P2 transporter
TbAT1-/-	P2 Transporter knockout
$t_{1/2}$	Half life
Tm	Melting temperature
VSG	Variant surface glycoprotein
WHO	World Health Organization

INTRODUCTION

A. INTRODUCTION

Human African trypanosomiasis, caused by *Trypanosoma brucei gambiense* and *T. b. rhodesiense*, is a devastating disease that has the potential to affect millions in sub-Saharan Africa (82). Treatment options for the disease are limited, as there are only four drugs available. All four compounds are associated with many problems, including cost, route of administration, increasing incidence of treatment failure, and reports of drug resistance (43, 51). Drug development efforts recently have focused on creating novel less toxic and more potent diamidine derivatives of pentamidine and on developing an orally active drug (80). A prodrug approach has been utilized to create orally available, lipophilic compounds which are transformed metabolically by cytochrome P450 (CYP450s) and cytochrome b5 enzymes to form active diamidines (65, 78). Pafuramidine, or DB289, the prodrug of DB75 (furamidine) is currently in Phase III clinical trials as an oral treatment of early stage trypanosomiasis.

To date, numerous diamidines and related compounds have been synthesized, many with potent anti-trypanosomal activity. Although several of these diamidines, such as DB75 and the closely related analog, DB820 are potent DNA binders, the overall mechanism of action of this series of compounds is unknown. Previous studies (3, 4, 50, 52, 60, 61) have established that the shape of the diamidine may affect the ability of the compounds to bind DNA, but it is not known whether this correlates with increased or decreased anti-trypanosomal activity. The compounds DB75 and DB820 are known to accumulate in several organelles, primarily in the DNA–containing nucleus and kinetoplast, but also in other organelles, such as the acidocalcisomes, which lack DNA.

Although the mechanism of action of diamidines is thought to be related to DNA binding, it is not known if diamidines like DB75 have targets in other organelles. The combined effects of modifying aromatic diamidine structures on intracellular localization and anti-trypanosomal activity has not been extensively investigated. The overall hypotheses for this research are that 1) diamidines are actively transported into trypanosomes where they exert their mechanism of action by interacting with DNA, and 2) that structural alterations lead to increased or decreased activity by altering both uptake and intracellular distribution in the trypanosomes. These hypotheses are based on three lines of evidence: 1) diamidine compounds bind DNA in AT rich regions of the minor groove (12), 2) structural modifications of diamidines have been shown to alter DNA binding and distribution in cancer cells (48), and 3) the recognition moiety for at least one known uptake transporter on the trypanosome surface includes a diamidine recognition site (24).

B. TRYPANOSOMIASIS: HISTORY AND CURRENT SITUATION

Human African trypanosomiasis, also known as African sleeping sickness, is a parasitic disease caused by *Trypanosoma brucei* subspecies *rhodesiense* and *gambiense.* Trypanosomiasis has affected sub-Saharan Africa for hundreds of years, with Atkins in 1721 and Winterbottom (in 1803) first describing the disease (22). However, it wasn't until the late 1800s–early 1900s that the causative agents of both the human infection and related animal infection were described. In 1894, David Bruce investigated an outbreak of the cattle wasting disease, nagana, in sub-Saharan Africa. Bruce identified trypanosomes in the blood of infected cows. He also experimentally determined that the trypanosomes isolated from infected cattle were able to cause nagana in other animals. Additionally, Bruce recognized that the tsetse fly (*Glossina* sp) could spread the disease, and noted nagana's similarity to the human disease known to cause lethargy. For his work, trypanosomes were later named after him (*Trypanosoma brucei*). In 1909, Freidrich Kleine demonstrated the essential role of the tsetse fly in the transmission of trypanosomiasis. Everett Dutton in 1902 identified the trypanosome that caused Gambian, or chronic trypanosomiasis (*Trypanosoma brucei gambiense)*. Finally, in 1910, J. W. W. Stephens and Harold Fantham described *T. b. rhodesiense*, the causative agent of the acute form of human trypanosomiasis (22, 38).

There have been several epidemics of human African trypanosomiasis (HAT) in Africa during the last century (82). Through surveillance, control and treatment efforts of the colonial powers, the disease was brought under control by the 1960s. However, once the colonies gained their freedom, control efforts lapsed and the disease began its resurgence in the 1970s (82).

In Angola for example, efforts to control and prevent trypanosomiasis began in 1901, and evolved into mobile teams in the 1940s to provide treatment and prophylactic services (71). This culminated in the development of the 'Brigade for Pentamidization' and the development of 26 health sectors, in certain regions of the country, in endemic areas affected by trypanosomiasis. By the time the country gained its independence in 1974, there were only 3 new cases detected in the country as a result of these control efforts (71). Unfortunately, the control infrastructure under the new government was never fully established and was impeded by lack of officers, equipment, vehicles and medical facilities. Outside support led to some work on trypansomiasis control. In the early 1990s, the program's capabilities were again destroyed by civil war, only to resume with the aid of outside agencies such as Médicins sans Frontières (MSF). Conflict in the late 1990s again impeded efforts to control trypanosomiasis, and the numbers of infected patients began to rapidly rise again (71). This pattern of control and resurgence of the disease has been repeated in many other newly independent African nations.

In the current epidemic, HAT has the potential to affect 60 million people in Sub-Saharan Africa. The disease is at epidemic levels in Angola, southern Sudan, and the Democratic Republic of Congo, and is highly endemic in countries such as Uganda. In some villages in these countries, the prevalence of trypanosomiasis is between 20 and 50%, with mortality higher than that of HIV/AIDS (46, 82). Through recent control efforts, surveillance has increased and the number of new cases reported between 1998 and 2004 for the disease decreased by 20,000 patients (82).

B.1. Trypanosomiasis Manifestations and Symptoms

Trypanosomiasis is a vector borne disease, with transmission occurring after the bite of an infected tsetse fly (*Glossina* sp.) The parasites *T. b. gambiense* and *T. b. rhodesiense* are morphologically identical parasites, although the disease manifests itself differently depending on the subspecies (79). The parasites also differ in terms of geographical location and reservoirs. It has long been thought that only *T. b. rhodesiense* had an animal reservoir, but *T. b. gambiense* has recently been shown to exist in 8 wild animal species (15). It is still unknown what role these species may play as a reservoir of the Gambian infection.

Gambian sleeping sickness is a chronic disease found primarily in central and western Africa, while the Rhodesian form of trypanosomiasis is an acute disease found in eastern and southern Africa (79). Both chronic and acute trypanosomiases are characterized by two stages of infection, although the overall length of the infection differs. Symptoms develop rapidly in *T. b. rhodesiense* infections, with symptoms of the second stage becoming apparent within weeks, whereas Gambian infections can persist for years in a subacute first stage before progressing to the second stage and death.

Symptoms of the early stage (also known as the hemolymphatic stage) of both forms of the disease are general, and include malaise, fever, and lymphadenopathy. The early stage of Gambian sleeping sickness is often asymptomatic for several months before proceeding to a subacute febrile period, which is then eventually succeeded by the late stage central nervous system (CNS) infection (51, 79) After the initial bite of the tsetse fly, trypanosomes disseminate from the lymphatic system into the bloodstream and extravascular spaces. In the early stage, trypanosomes are confined there and proliferate with waves of parasitemia, and manage to evade the host immune system by switching the variant surface glycoprotein

(VSG) antigen covering the surface of the parasite (45, 75). The VSG antigen is the main surface protein found on the bloodstream trypanosome, and there are approximately 1000 genes that encode VSGs (76). As the disease progresses, the parasites are able to penetrate the blood brain barrier and invade the CNS. Symptoms of the late CNS infection include meningoencephalitis, sensory disturbances and hallucinations, dementia, and reversal of the sleep cycle. If untreated, the patient lapses into a coma and dies (33, 79).

B.2. Diagnosis and Staging of Trypanosomiasis

Diagnosis of sleeping sickness is made primarily through several screening processes. The clinical presentations of early stage trypanosomiasis are often vague and variable, and the disease may be misdiagnosed. The presence of a chancre or enlarged cervical lymph nodes (Winterbottom's sign) are clinical signs that are indicative of trypanosomiasis. Suspected trypanosomiasis can be confirmed using serological and parasitological techniques. The Card Agglutination Trypanosomiasis Test (CATT) is often used on whole blood as a preliminary screen to diagnose trypanosomiasis. If the CATT is positive, the diagnosis is confirmed by visualizing trypanosomes in blood, lymph, or CSF (cerebrospinal fluid). With the Gambian form of the infection, parasitemia is often very low, and blood must be concentrated to determine if trypanosomes are present. The mini anion-exchange centrifugation test (mAECT) and capillary tube centrifugation test (CTC) are both used to concentrate trypanosomes in blood (1, 6, 43). In the future, polymerase chain reaction, or PCR-based tests may be useful for the detection of trypanosomiasis in patients. One of these techniques is a PCR-oligochromatography dipstick test, HAT-PCR-OC (29). This test combines PCR and membrane chromatography to detect DNA, and is a very rapid test (<5 min). The HAT-PCR-OC test was consistently able to detect one parasite in 180 μl of blood,

or 5 fg of trypanosome DNA (29). Additionally, the test does not cross react with other Kinetoplastid species and is specific for *Trypanozoon* species (29). However, the feasibility of using this test in the clinic has not been investigated.

As treatments for trypanosomiasis are stage specific, it is important to determine what stage of infection patients have. This is done by determining the presence of trypanosomes and white blood cells in the CSF. The presence of trypanosomes in the CSF is indicative of late stage, or CNS infection. However, if trypanosomes are not found in the CSF, the presence of greater than 5 leukocytes/mm^3 has historically been used to indicate second stage trypanosomiasis (43). Recently, the MSF programs operating in sub-Saharan Africa increased the threshold for determining late stage infection to 10 leukocytes/mm^3. This was done for several reasons, including evidence that pentamidine could be effective against patients with "intermediate" CSF white blood cell counts (6-10 cells/mm^3). This was also done to reserve the use of the very toxic drug, melarsoprol, the first line treatment for late stage HAT, in cases that could be cured with pentamidine (6). However, preliminary results do show that treating patients with intermediate CSF white cell counts between 6-10 cells/mm^3 with pentamidine, does lead to three times the treatment failure than those with CSF white cell counts between 0-5 cells/mm^3 (6). In this investigation, the relapse rate was still only 5% overall, which is an acceptable level of treatment failure (6).

A dot – ELISA test has been developed for staging of trypanosomiasis (21). This test detects anti-neurofilament and anti-galactocerebroside proteins and cross-reacts with invariant membrane antigens of *T. brucei.* Detection of these antibodies in CSF is characteristic of second stage infection (21). The dot – ELISA test showed 80% sensitivity and 100% specificity in field testing (21). This high level of sensitivity and specificity make

this test an excellent potential staging test, especially when patients have intermediate white blood cell counts in the CSF (21). The test must be further investigated on a larger scale to verify its specificity and accuracy.

C. *TRYPANOSOMA BRUCEI*

T. brucei is a eukaryotic parasite belonging to the order Kinetoplastidia (56). Trypanosomes are digenetic organisms that progress through a life cycle as they are transmitted between vector and host (Fig 1.1) Trypanosomes are transmitted between hosts by the tsetse fly (*Glossina* sp) (55). The trypomastigote ingested by the tsetse fly blood meal, transforms into a procyclic trypomastigote. The major protein that makes up the surface coat of the trypanosome is changed from the VSG into procyclin. Additionally, the mechanism for energy generation is switched from primarily glycolysis to a mitochondrion based respiratory system, along with the necessary changes to the mitochondrion structure and metabolic activation. The procyclic trypanosome proliferates in the tsetse fly midgut before moving to the salivary gland. There the trypanosome undergoes further differentiation into a non-proliferating, VSG expressing, metacyclic bloodstream form (55). The metacyclic trypanosome is injected into a host organism when the tsetse fly takes its blood meal. The injected trypanosome differentiates into a slender bloodstream trypomastigote that relies on glycolysis for energy generation. As trypanosomes proliferate in the bloodstream, they are transformed into non-proliferative short, stumpy forms. The short stumpy forms limit trypanosome number and prolong host survival, which therefore increases the chance for transmission of the infection (55).

All cellular organelles are present in both the procyclic (the vector, or fly form) and the bloodstream (infected host) form, although location of organelles within the cell may differ. In addition to the characteristic organelles of eukaryotes, including the nucleus, mitochondria, lysosomes, Golgi apparatus and endoplasmic reticulum (ER) (37), trypanosomes have specialized organelles and structures, such as the acidocalcisomes (31)

and kinetoplast (35), as seen in Figure 1.2, and glycosomes (57). Unique organelles found in trypanosomes could be potential drug targets since humans lack equivalent structures, for the most part.

The acidocalcisomes, as the name suggests, are acidic calcium storage organelles found in many microorganisms, including eukaryotic parasites such as *T. brucei* and *T. cruzi*, *Plasmodium spp.,* and *Toxoplasma spp*. Acidocalcisomes sequester ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Na^{+}, and phosphorous in the form of polyphosphates and pyrophosphates (32). Polyphosphates accumulate in acidocalcisomes at millimolar to molar levels with an average phosphate chain length of 3.2 phosphates (31).

On the surface of acidocalcisomes are several pumps and exchangers which concentrate ions in the organelle, including a vacuolar - H^{+}-ATPase, a vacuolar - H^{+}-pyrophosphatase (PPase), a Ca^{2+}-H^{+} - ATPase, a Ca^{2+}/H^{+} - exchanger and a Na^{+}/H^{+} - exchanger. Many of these pumps and exchangers can be inhibited, resulting in the release of acidocalcisome contents into the cytosol (31). The physiological role of acidocalcisomes is not known, but hypotheses include serving as an energy or calcium store for the parasite, and involvement in osmoregulation and pH homeostasis (32). All three of these functions could explain why long and short chain polyphosphates accumulate in high levels in acidocalcisomes (32). Recently in *T. cruzi* and other parasites, the acidocalcisomes have been implicated in an efflux pathway linked to the flagellar pocket. This efflux pathway is involved with regulatory volume decreases and the release of molecules from the parasites (64). Recent evidence also suggests that acidocalcisomes are conserved from prokaryotes to eukaryotes – including humans. Dense granules that are similar to acidocalcisomes have recently been characterized in human platelets. These granules are morphologically and

structurally similar to acidocalcisomes, and contain high levels of polyphosphates released after thrombin stimulation (31). Acidocalcisome-like organelles have not been found in other human cells yet.

The kinetoplast is a unique DNA structure found in the single mitochondrion of the trypanosome (68). Kinetoplast DNA (kDNA) is comprised of circles of DNA – about 50 maxicircles and about 10000 minicircles – that are interlocked into a disc – like network of about 10^7 kDa (68). Maxicircles encode for many of the necessary enzymes and proteins for the mitochondrion, while the minicircles encode guide RNAs (gRNA), which are involved in RNA editing processes that occur within the trypanosome mitochondrion (68). Guide RNAs correct mRNA gene transcripts by inserting or deleting uridine molecules in the transcript to create a functional mRNA. The kinetoplast is connected to the flagellar basal body of trypanosomes (68), and the mitochondrion extends throughout the organism. The function of the mitochondrion changes as the trypanosome proceeds through its life cycle (57, 68), especially in terms of energy metabolism. Unlike the procyclic form found in the tsetse fly, the mitochondrion of the bloodstream form trypanosome has poorly developed cristae (68), and contains neither enzymes involved in the tricarboxylic acid cycle , or TCA cycle nor a cytochrome - containing respiratory chain (57). Differences in the respiration and metabolic processes between human and trypanosome cells could likely be future targets for the development of antitrypanosomal drugs.

Glycosomes are peroxisome-related organelles in which carbohydrate metabolism is compartmentalized. In the bloodstream form of the trypanosome, the first seven steps of glycolysis occur in the glycosome, with the remaining conversions occurring in the cytosol (67). Bloodstream trypanosomes depend entirely on glycolysis for the production of ATP.

This form of the trypanosome is able to produce both pyruvate and glycerol from glucose (57). Although the majority of the enzymes found in the glycosomes of bloodstream trypanosomes are related to glycolysis, the organelle is also involved in other metabolic processes throughout the trypanosome life cycle (57). Inhibition of any of these metabolic processes could be potential drug targets.

D. TREATMENT OPTIONS

Current therapy for trypanosomiasis is limited to four drugs (Figure 1.3), all of which are problematic. Among the problems are toxicity, lack of an oral dosage form, lack of drug availability, high cost, and increased incidence of treatment failures and reports of drug resistance (51). Drug therapy is especially problematic in rural Africa where trypanosomiasis occurs, due to the realities of field medicine in nations where the annual per capita health budget is less than $10 (82). Treatment is dependent upon the parasite subspecies, and the clinical stage of infection. Two drugs, pentamidine and suramin (Fig 1.3) are unable to cross the blood brain barrier, and are therefore inactive against secondary trypanosomiasis (43, 82). The other two drugs used to treat trypanosomiasis, melarsoprol and eflornithine, are able to cross the blood brain barrier.

D.1. Early Stage Treatment – *T. b. gambiense*

Pentamidine, an aromatic diamidine first used in 1940, is the first line treatment for early stage *T. b. gambiense* infections (51). It has also been used as a treatment for early stage Rhodesian trypanosomiasis, but produces variable results (1). As a hydrophilic dication, it is unable to pass the blood brain barrier, and is therefore ineffective against the CNS stage of infection (34). Adverse effects of pentamidine include hypotension, hepatotoxicity, renal failure and hypoglycemia that can lead to diabetes (51). Pentamidine is administered through a series of intramuscular (IM) injections at a dose of 4 mg/kg/day for a total of 7-10 injections. Due to the compound's long elimination half-life, pentamidine has in the past been used as a chemoprophylaxis for trypanosomiasis (1). The elimination half-life of pentamidine administered to trypanosomiasis patients has been calculated to be between 22 and 47 h (13).

The exact mechanism of action of pentamidine remains unknown, but several hypotheses exist. These include binding to DNA, and the linearization and destruction of the circular DNA in the kinetoplast (69, 70). Other authors theorize that the generation of dyskinetoplastic trypanosomes in the infected host would not be enough to kill the organisms, and that another mechanism must exist (77). Additionally, pentamidine has been shown to reversibly inhibit S-adenosylmethionine decarboxylase (SAMDC) and may play a role in inhibiting polyamine metabolism in the trypanosomes (10, 11).

Pentamidine accumulates in trypanosomes via uptake by three transporters on the surface of the cell: a High Affinity Pentamidine Transporter (HAPT1), a Low Affinity Pentamidine Transporter (LAPT1), and the P2 transporter (24). Trypanosomes are scavengers in the bloodstream of infected hosts. They require uptake of many biosynthetic precursors, such as purine bases and nucleosides, including adenine and adenosine. Several transporters have been discovered that actively transport adenine, adenosine and other purines into trypanosomes. These include the P2 and P1 transporters. The P1 transporter exists in the procyclic and bloodstream forms, while the P2 transporter is found only in bloodstream form trypanosomes. The recognition site for the P2 transporter appears to interact with the nitrogen at position 1 and the amine group at position 6 of adenosine, as seen in Figure 1.4. This $H_2N\text{-}C(R_1)\text{-}NR_2$ motif is found in diamidine compounds and melaminophenyl arsenicals such as melarsoprol (Figure 1.3). Pentamidine however, is also taken up by additional transporters, the HAPT1 and LAPT1. Uptake of pentamidine through HAPT1 is inhibited only by propamidine, while LAPT1 uptake is not inhibited by any other known diamidine compound, suggesting selectivity for pentamidine uptake (23). No endogenous substrates for LAPT1 and HAPT1 have been discovered. The presence of three

pentamidine transporters also suggests that transport-related resistance to pentamidine would be slow to develop, whereas compounds that are only substrates for one transporter, such as melarsoprol, might develop resistance much faster. This has been seen in the field, where despite extensive use as a prophylactic and treatment for 60 years, trypanosomes have not developed resistance to pentamidine (23). Other compounds such as melarsoprol for human trypanosomiasis and diminazene for treatment of animal trypanosomiasis, which are taken up by only one or two transporters, have generated resistant trypanosomes with extensive use (23).

D.2. Early Stage Treatment – *T. b. rhodesiense*

For early stage *T. b. rhodesiense* infections, the polysulfated napthylamine compound, suramin (Figure 1.3), is the drug of choice. Suramin is a high molecular weight anion. Like pentamidine, it is unable to cross the blood brain barrier (34, 51). Suramin, which has been used as a treatment since the 1920s, has also been associated with severe side effects including renal failure and anaphylactic shock (51). Suramin is administered by intravenous (IV) injection at a typical dose of 20 mg/kg, with no more than 1 g administered per injection. However, due to the risk of shock, a test dose (0.1 g) must be administered before treatment begins. The treatment is 5-6 injections administered at intervals of 5-7 days (1, 11). Suramin is transported into trypanosomes by pinocytosis, or receptor mediated endocytosis when bound to serum proteins (77). Suramin is believed to inhibit several glycolytic enzymes in trypanosomes. The drug exerts a slow trypanocidal effect, most likely because it cannot enter the glycosome compartment that contains the glycolytic enzymes, but instead, can only inhibit the enzymes when they are found in the cytoplasm while they are

transported between organelles after synthesis and modification (77). Some reports do indicate that suramin acts synergistically with CNS treatments, such as melarsoprol (11).

D.3. Late Stage Treatment

D.3.a. Melarsoprol – *T. b. gambiense* and *T. b. rhodesiense*

Melarsoprol is a melamine-based organic arsenical compound that has been used for treating both *T. b. gambiense* and *T. b. rhodesiense* CNS infections since the 1940s (51). Melarsoprol is very toxic, and causes a fatal reactive encephalopathy in 5-10% of patients. Additionally, melarsoprol has also been associated with increasing incidence of treatment failure (51). Historically, melarsoprol has been administered using a 26 day treatment schedule (3-4 series of 3-4 injections are administered in increasing doses, interspaced with resting periods of 7-10 days). A 10-day melarsoprol regimen recently has been proven to be equally efficacious against *T. b. gambiense* CNS infections (15, 66). The shorter treatment regimen has several advantages including less drug used, shorter hospitalization requirements, and increased treatment compliance (15, 66).

Melarsoprol is believed to kill trypanosomes by inhibiting glycolytic enzymes. Melarsoprol has been shown to inhibit pyruvate kinase, phosphofructokinase, and fructose-2,6-bisphosphatase (77). The inhibition of glycolysis leads to lysis of the cells, also seen with suramin (77). Melarsoprol has also been shown to form a stable adduct with trypanothione, a cofactor similar to glutathione that is involved in the redox balance of trypanosomes. Whether this plays a role in its mechanism of action still remains to be determined (77).

There are several potential routes of entry into trypanosomes for melarsoprol. The lipophilic compound may be able to passively diffuse across the trypanosome membrane

(23). Melarsoprol is also accumulated in trypanosomes through the P2 transporter. Prevention of uptake could play an important role in the increased rates of melarsoprol resistance seen in the clinic. Point mutations of the P2 transporter gene, or *TbAT1* gene, have been associated clinically with melarsoprol resistance (23). When a knockout of the P2 transporter in trypanosomes was developed, however, melarsoprol resistance was only about 2-3 fold over the wild type strain. This indicates the potential for other mechanisms of uptake into trypanosomes (54), which may include passive diffusion as well as transport mechanisms that may be upregulated in the absence of the P2 transporter. The P1 transporter has been shown to be upregulated in the *TbAT1-/-* line (36), and the same phenomena may be seen with other transporters.

D.3.b. Eflornithine – *T. b. gambiense*

The only new drug to be introduced for the treatment of trypanosomiasis in almost 50 years is eflornithine (DL-α-difluoromethylornithine), or DFMO, which was first used for trypanosomiasis treatment in the 1980s (51). Eflornithine was initially developed as an anticancer therapy. Eflornithine is used for late stage Gambian infections; it is inactive against the Rhodesian form of the disease. Adverse effects associated with eflornithine include panocytopenia, diarrhea, convulsions and hallucinations (16). Although eflornithine has sufficient lipophilicity for use as an oral treatment, oral administration has been associated with higher rates of relapse and increased reports of adverse effects (51). Like the other drugs, eflornithine is currently administered parenterally. It is administered in a series of 56 intravenous infusions. Recently, MSF has recommended that due to high relapse rates with melarsoprol, eflornithine be used as the first line treatment for all late stage cases of trypanosomiasis in the Republic of Congo (5).

Eflornithine is a suicide inhibitor of ornithine decarboxylase (ODC), an essential enzyme in the polyamine biosynthetic pathway (11, 77). ODC is an essential enzyme which leads to the formation of essential polyamines such as putrescine, spermidine, and spermine (77). Polyamines are vital for replication of trypanosomes (17). DFMO is only effective against *T. b. gambiense* due to the slow turnover rate of the ODC enzyme in this parasite compared to the human form of the enzyme (23). Eflornithine is inactive against *T. b. rhodesiense* because in this subspecies, the enzyme has a half – life of only 4.3 h compared to >18 hr for Gambian subspecies (23). Inactivation of the ODC enzyme leads to the transformation of bloodstream trypanosomes to a short metacyclic form that is unable to divide and alter the surface antigen VSG. These changes allow the host immune system to more effectively attack the invading parasite and clear parasitemia (11, 30).

D.3.c. Combination Therapy for Late Stage Treatment

A recent clinical trial investigated the potential for using combination therapy to treat late stage trypanosomiasis, due to the toxicity of melarsoprol and the long, costly administration of eflornithine (63). This study investigated combinations of melarsoprol + eflornithine, melarsoprol + nifurtimox, and eflornithine + nifurtimox. Nifurtimox (Figure 1.3) is an oral treatment used for Chagas' disease, caused by *Trypanosoma cruzi*. Nifurtimox toxicity includes neurological and gastrointestinal disorders that increase with the length of therapy. Nifurtimox is not registered for treatment of HAT, but has been used for compassionate treatment (63). In the trial, the dosing regimens of all three drugs were shortened. The authors found that the combination of melarsoprol and nifurtimox resulted in higher than expected fatalities, with 4/18 patients dying of reactive encephalopathy during treatment, and a less than 50% cure rate over 24 months. The eflornithine + nifurtimox

group, on the other hand, was very effective, with a greater than 94% cure rate over the 24 month observation period. The combination of eflornithine, a trypanostatic drug and the trypanocidal nifurtimox would thus appear to be efficacious. Additionally, nifurtimox is relatively cheap, and the combination therapy requires only half of the normal dose of eflornithine to be administered, which reduced side effects as well as cost (63). However, the combination still needs to be evaluated in more patients.

D.4. Need for New Therapies

There is still a great need for the development of new therapies for African trypanosomiasis. The four drugs currently used are old, expensive and difficult to administer, in addition to toxicity and drug resistance issues. The drug supplies are currently ensured, due to a five-year agreement was made between the World Health Organization, or WHO, and Aventis in 2001. In this agreement, Aventis donated $25 million dollars to support distribution of pentamidine, melarsoprol and eflornithine (81). Bayer also provides suramin to the WHO free of charge. Recently, due to the success of the program in controlling trypanosomiasis, the pharmaceutical companies have agreed to extend their support of WHO efforts to treat and prevent trypanosomiasis (82). Only one drug for trypanosomiasis, eflornithine, has made it to market in the past 30 years, primarily because it was initially developed as a cancer therapy (58). Few drugs since then have entered clinical trials. Megazol, a nitroheterocyclic compound, a potent antitrypanosomal compound in vitro and in vivo, was being developed in recent years. However, due to genotoxicity studies, development of the drug was not pursued (11). Currently, DB289, or pafuramidine, which is being developed as an oral treatment for trypanosomiasis, is enrolled in Phase III clinical trials in Africa. DB289 is the prodrug of DB75 (furamidine), an active diamidine analog of

pentamidine (Fig 1.4). The compound is only active against the early stage of the disease, and has little effect against chronic trypanosomiasis in animal models (43). Therefore, it is essential that drug development continue until effective, cheap treatments are available for both early and late stage trypanosomiasis.

E. Development of Novel Diamidine Compounds and Prodrugs

Pentamidine is a diamidine compound that has been shown to be effective in vitro and in vivo (in vitro IC_{50} = 1.1 nM and in vivo ED_{50} = 3.2 μmol/kg against S427 *T. b. brucei*) for animal models of trypanosomiasis. However, the drug is associated with many toxic effects, including hypoglycemia, liver toxicity, pruritis, rash, vomiting, and renal failure (11). Some of these toxicities are believed to be related to metabolism of the compound. Pentamidine has been shown to form at least seven phase I metabolites and several phase II metabolites. Metabolism appears to decrease the efficacy of the compound against microbes and several of the metabolites appear to be more toxic than pentamidine (7, 8, 14, 19, 20, 44). A series of novel diamidines have been synthesized, many of which have good activity against trypanosomes in vitro (12, 39-41). However, like pentamidine, other aromatic diamidines are unable to efficiently cross membrane barriers, and thus have poor oral bioavailability. Oral administration would be of great advantage for the treatment of trypanosomiasis, given the many problems with parenteral administration in Africa. Trypanosomiasis is primarily a rural disease and is found in many of the poorest nations in Africa. Rural areas are not always adequately equipped to perform multiple sterile injections or long term infusions (43).

Two lead compounds (Figure 1.5) have been identified from this series of pentamidine analogues, DB75 (2,5-bis(4-amidinophenyl)-furan) and DB820 (6-[5-(4-carbamimidoyl-phenyl)-furan-2-yl]-nicotinamidine). In animal models of acute trypanosomiasis, both of these compounds show efficacy against trypanosomes. DB75 and DB820, while potent anti-trypanosomal agents (53), are unable to effectively cross membrane barriers such as the gut or blood brain barrier (83); therefore, a prodrug approach was utilized to develop prodrugs with sufficient lipophilicity to penetrate barriers (DB289 is

the prodrug for DB75, and DB844 for DB820). DB289 and DB844 are methoxime prodrugs of DB75 and DB820 – i.e. hydrogen molecules on the terminal amidines have been replaced with a methoxy group (-OCH_3) (Figure 1.5). Prodrugs exhibit poor activity in vitro, reinforcing the requirement of an in vivo transformation to target parasites. A more lipophilic prodrug will be able to be administered orally and be absorbed from the intestinal lumen. The compound can then disseminate through the body, where it will be metabolized to the active diamidine compound necessary for killing trypanosomes.

The drug development efforts of the UNC consortium have concentrated on aromatic diamidine compounds. These compounds are synthesized at UNC and Georgia State University. A library of more than 2000 compounds exists and contains active diamidines and their prodrugs. Diamidines and prodrugs are tested in vitro and in vivo in models for trypanosomiasis as well as other parasitic diseases (40, 42, 72).

E.1. DNA Binding and the Mechanism of Action of Diamidine Compounds

Diamidine compounds such as pentamidine have a long history of use as antimicrobial agents, especially for the treatment of various human parasitic diseases, including *Pneumocystis* pneumonia and trypanosomiasis (80). Pentamidine and analogs such as DB75 and DB820, also bind to AT rich regions of the minor groove of DNA (12, 18, 50, 59). Analogs that have a single amidine moiety (monoamidines) are unable to bind to the minor groove, and exhibit poor anti-trypanosomal activity (12). Although it is known that diamidine compounds bind to the minor groove of DNA with varying affinities, the exact mechanism of action of killing remains unknown. For example, compounds could interfere with transcription, or could inhibit DNA dependent enzymes (12). Additionally, a compound could selectively target DNA in either the kinetoplast or nucleus, with little effect on other

organelles. The possibility also exists that there are multiple mechanisms of action of diamidine compounds, including some that may not involve DNA binding at all (62).

The effect of diamidine substitution could also affect the mechanism of action of compounds. For example, a phenyl substituted analog of DB75, DB569, has similar DNA binding properties to DB75. Both compounds bind to the minor groove of AT rich regions with similar affinity ($K > 10^7$) (48). Studies in tumor cell lines have shown markedly different intracellular localization, however, with DB75 localizing in the nucleus, and DB569 localizing in cytoplasmic granules. This study concluded that substitution of diamidine with a phenyl ring, regardless of core structure of the compound, was the only substitution that shifted intracellular distribution from the nucleus to the cytoplasm (48). This shift did not greatly affect the cytotoxicity of DB569 when compared to DB75 (48). The in vitro IC_{50} values for DB569 against STIB 900 (*T. b. rhodesiense)* and S427 (*T. b. brucei)* parasites is much higher than DB75 – but it is unknown whether this is related to differences in intracellular distribution of the compounds in trypanosomes. DB569 has been shown to have increased activity compared to DB75 against *T. cruzi* and *Leishmania* parasites (26), but both compounds appear to have similar intracellular distribution in these parasites. The hypothesis of differential distribution for DB569 in *T. brucei* parasites cannot be ruled out as of yet, since both of the organisms are quite different than *T. brucei*, despite belonging to the same order. The possibility exists of more than one mechanism of action for diamidines, which could be manifested in different distribution within trypanosomes.

E.2. Selective Uptake into Trypanosomes

Selective uptake of diamidines into trypanosomes is likely important to their mechanism of action. As mentioned previously, diamidines have been shown to accumulate

in trypanosomes by three transporters so far, the HAPT1, LAPT1, and P2 transporter (24). Diamidines have also been shown to potently inhibit uptake of adenosine by the P2 transporter (25, 49). Selective uptake into trypanosomes would not only enhance efficacy, but also reduce toxicity, due to less being taken up by host cells. To this end, there are several efforts underway to develop anti-trypanosomal drugs that are selectively accumulated in trypanosomes. One effort has focused on the development of compounds that have benzamidine or melamine moieties, but this approach has not yet yielded very potent anti-trypanosomal compounds (73, 74). Aromatic diamidines, having a benzamidine moiety on either end, also appear to accumulate in trypanosomes by one or more transporters. The ideal diamidine would be transported, like pentamidine, into the trypanosome by multiple transporters, in order to prevent transporter related drug resistance.

F. Rationale for Proposed Studies

There is a great need for new treatments of human African trypanosomiasis and other parasitic diseases worldwide. Major pharmaceutical companies often neglect drug development for diseases of the developing world, such as sleeping sickness. Although the consortium's approach to the development of novel therapies has generated many dicationic compounds with anti-parasitic activity, there is little known about the exact mechanisms of action. Currently, a library of greater than 2000 compounds exists, with compounds synthesized at both UNC and GSU. Many researchers have proposed mechanisms of action for diamidine compounds through the years, with the primary theories being DNA binding, linearization of the kinetoplast DNA structure, and interfering with RNA editing (11, 69). Knowledge of the intracellular distribution of compounds could provide insight into possible mechanisms of action. Fluorescent compounds like the ones available in the aromatic diamidine libraries can be especially helpful for identifying unknown potential mechanisms of action or loss in the trypanosomes. Some of the compounds investigated in this dissertation have been examined in cancer cells with fluorescence microscopy to visualize distribution (47, 48), to determine mechanism of action or loss. Although these studies with select compounds have investigated multiple compounds in a series (47, 48), to our knowledge these studies have not been performed with *T. brucei* strains.

As seen with cancer cells, the structure of potential anti-trypanosomal compounds could also affect uptake and accumulation into trypanosomes, in addition to the distribution of the compounds within the organism. Aromatic diamidines may be taken up by any one of three transporters in the bloodstream form trypanosome, and substrate recognition may be affected by structure modifications. This could lead to alternative uptake mechanisms, and

therefore, increased or decreased efficacy. The number of transporters utilized for compound uptake could also affect the potential for drug resistance, as seen with pentamidine and other diamidines (54). An overall goal for development of diamidine compounds is to develop a compound that displays the uptake properties of pentamidine, but is less toxic. Knowledge of the effects of structure on the mechanism of action, uptake and distribution, and anti-trypanosomal activity could lead to the development of more potent molecules in the future. The effect that structures have on intracellular distribution could explain why some compounds are not efficacious against trypanosomes. Many compounds that have been synthesized by the Boykin and Tidwell laboratories also have a lack of correlation between in vitro and in vivo activity, which could be related to uptake or distribution. The studies outlined here could determine the cause of activity or the lack thereof. Many studies in the past have investigated the interaction between structure and anti-trypanosomal activity of investigational compounds (2, 9, 26-28, 39-41), but few studies have investigated the intracellular distribution of the compounds, which may be essential to activity. If a compound does not reach the target of interest in the cell, anti-trypanosomal activity will be poor. The investigations outlined in this proposal will incorporate studies of multiple aspects of the anti-trypanosomal activity – uptake, distribution, and accumulation, and the relationship these have to compound structure.

The major hypothesis governing the experiments in this dissertation is that **diamidine compounds rapidly accumulate in trypanosomes via uptake transporters such as the P2 transporter and distribute to the DNA containing kinetoplast and nucleus**. In order to

investigate this hypothesis, accumulation and distribution of diamidines in trypanosomes was evaluated through the following specific objectives:

1. Determine the accumulation and intracellular distribution patterns of DB75 and DB820, two lead compounds for the treatment of HAT in African trypanosomes. Hypothesis: DB75 and DB820 are selectively accumulated in trypanosomes, and are localized to the nucleus and kinetoplast, the DNA containing organelles (Chapter 2).
    a). Determine in vitro activity and in vitro accumulation of DB75 and DB820 in African trypanosomes.
    b). Determine in vivo accumulation and distribution of DB75 and DB820 in trypanosomes isolated from infected rodents.
2. Investigate accumulation and distribution of DB75 in wild type and resistant strains of S427 *T. b. brucei*. Hypothesis: Accumulation of DB75 in the 'resistant' strains is less than in the wild type strain. Distribution will be the same, but will take longer to equilibrate into compartments (Chapter 3).
    a). Determine in vitro activity of DB75 in wild type (S427), *TbAT1-/-* (P2 transporter knockout), $DB75^{R}$-CL1 strain (DB75 resistant strain), and B428 (pentamidine resistant *TbAT1-/-*).
    b). Determine in vitro accumulation and distribution of DB75 in all four strains over time.
    c). Determine in vivo accumulation and distribution of DB75 in all four strains of trypanosomes over 8 hours.

3. Identify differences in in vitro accumulation and distribution of diamidine analogues of DB75 and DB820. Hypothesis: Compounds with a higher IC_{50} value accumulate in trypanosomes less than more potent compounds. Less potent compounds do not distribute to the proposed target organelles (i.e. the DNA containing nucleus and kinetoplast) (Chapter 4).

   a). Determine in vitro activity of analogues of DB75 and DB820 in African trypanosomes.

   b). Determine DNA binding affinity of analogues of DB75 and DB820.

   c). Determine in vitro accumulation and distribution of analogues of DB75 and DB820 in African trypanosomes (Chapter 4).

   d). Investigate in vitro activity and intracellular distribution of other diamidine analogues – the benzofurans and benzimidazoles (Chapter 5).

In addition to the above studies, experiments investigating the effects of temperature on distribution and accumulation of DB75 and DB249 in trypanosomes are presented in Appendix I. Appendix II presents preliminary data for the isolation of trypanosome organelles. Potential implications, future studies, and the conclusions drawn from this research are presented in Chapter 6.

Figure 1.1: Life cycle of *Trypanosoma brucei,* the African trypanosome (Image obtained with permission from CDC Public Health Image Library).

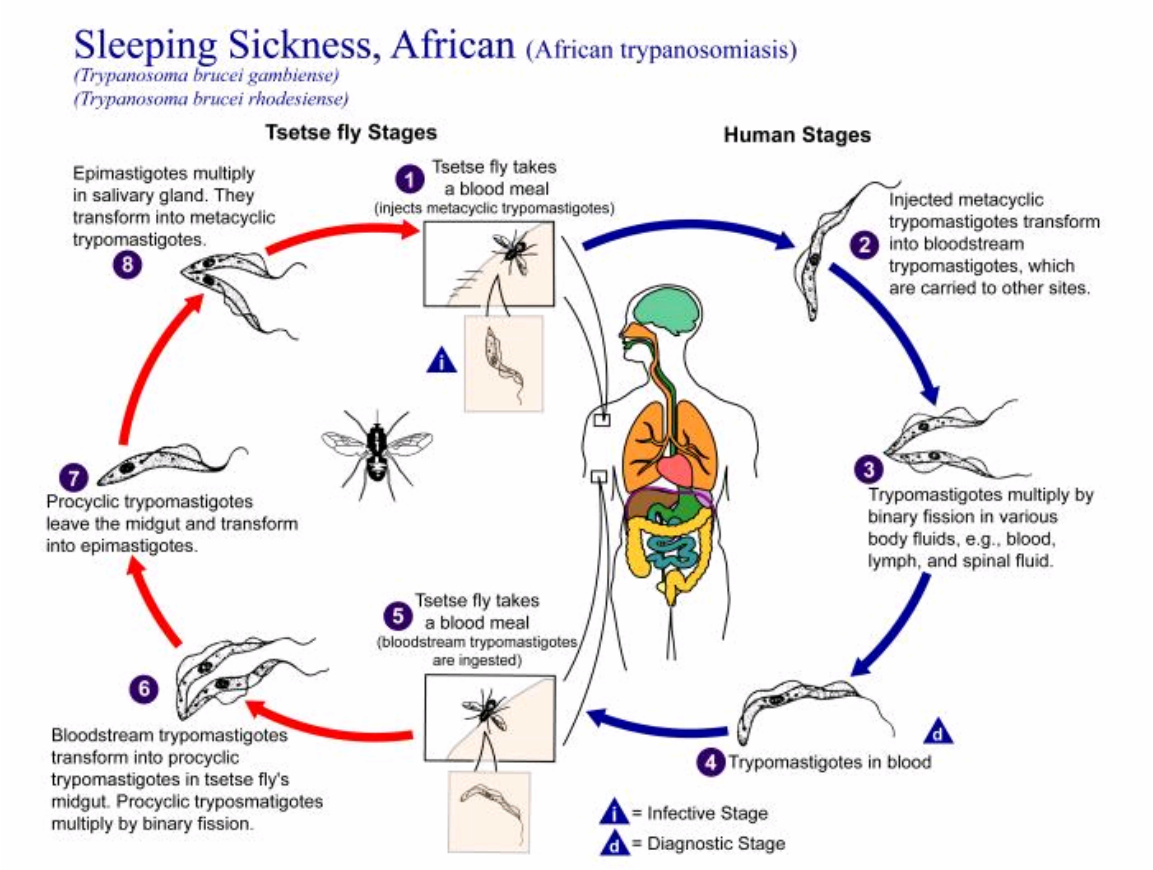


Figure 1.2: Diagram of trypanosome and organelles, modified from Grünfelder et al. 2003 (37).

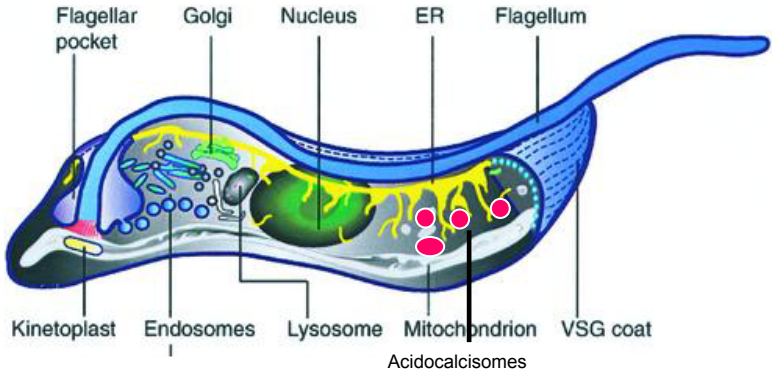
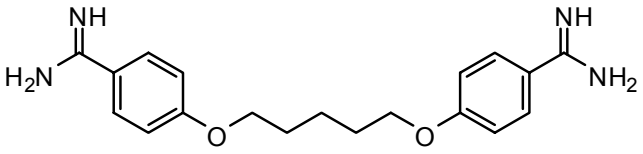
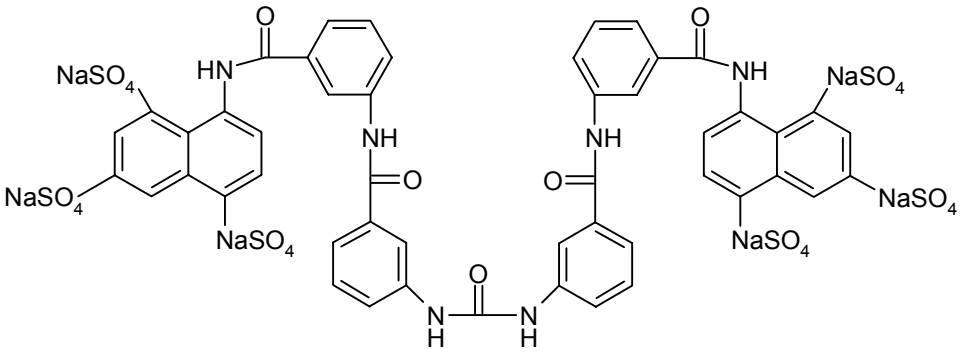


Figure 1.3: Structure of currently used treatments for human African trypanosomiasis.

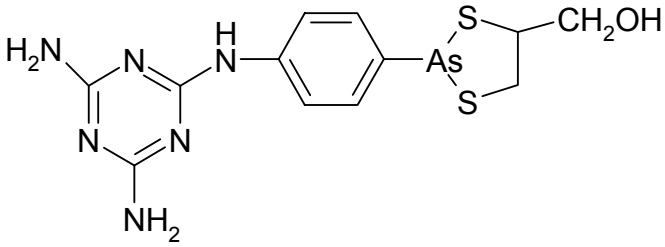
Pentamidine



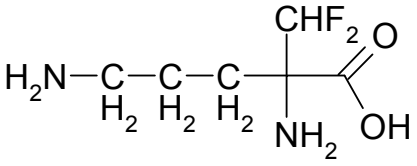
Suramin



Melarsoprol



Eflornithine



Nifurtimox

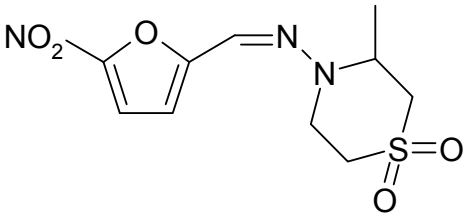


Figure 1.4: Recognition site for the P2 transporter.

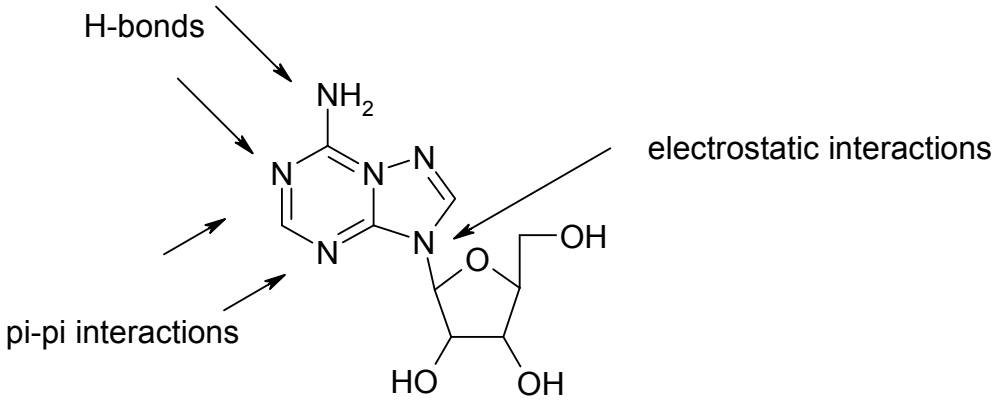
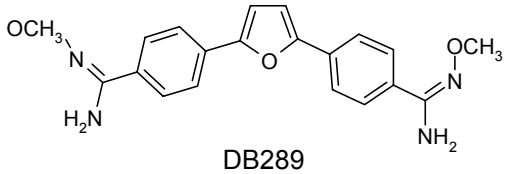
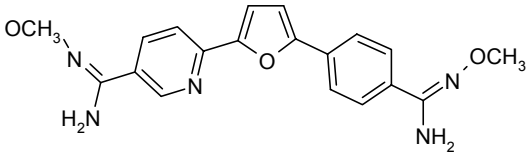


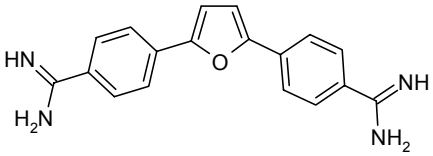
Figure 1.5: Structure of DB75 and DB820, and their prodrugs, DB289 and DB844.



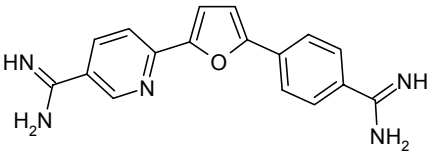
DB289
(pafuramidine)



DB844



DB75



DB820

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CHAPTER 2

ACCUMULATION AND INTRACELLULAR DISTRIBUTION OF THE ANTI-TRYPANOSOMAL DIAMIDINE COMPOUNDS DB75 AND DB820 IN AFRICAN TRYPANOSOMES

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A. Abstract

The aromatic diamidine pentamidine has long been used to treat early stage Human African trypanosomiasis (HAT). Two analogs of pentamidine, DB75 and DB820, have been shown to be more potent and less toxic than pentamidine in murine models of trypanosomiasis. The diphenyl furan diamidine, DB75, is the active metabolite of the prodrug DB289, which is currently in Phase III clinical trials as a new orally active candidate drug to treat first stage HAT. The new aza analog, DB820, is the active diamidine of the prodrug DB844, currently undergoing preclinical evaluation as a new candidate to treat CNS HAT. Exact mechanisms of anti-trypanosomal activity of aromatic dications remain poorly understood, with multiple mechanisms hypothesized. Pentamidine is known to be actively transported into trypanosomes and binds to DNA within the nucleus and kinetoplast. A long hypothesized mechanism of action has been that DNA binding leads ultimately to interference with DNA-associated enzymes. Both DB75 and DB820 are intensely fluorescent, which provides an important tool for determining the kinetics of accumulation and intracellular distribution in trypanosomes. We show in the current study that DB75 and DB820 rapidly accumulate and highly concentrate within trypanosomes, with intracellular concentrations over 15,000 fold higher than mouse plasma concentrations. Both compounds initially accumulate in the DNA-containing nucleus and kinetoplast, but at later time points concentrate in non-DNA-containing cytoplasmic organelles. Analysis of the kinetics of uptake and intracellular distribution are necessary to begin to define anti-trypanosomal mechanisms of action of DB75, DB820 and other aromatic diamidines.

B. Introduction

Human African trypanosomiasis (HAT), a devastating disease caused by *Trypanosoma brucei gambiense* and *T. b. rhodesiense*, has the potential to affect 60 million people in sub-Saharan Africa (27). HAT has two stages: an early stage, in which trypanosomes are confined to the bloodstream and other extracellular fluids; and a late stage in which trypanosomes are able to cross the blood brain barrier and invade the central nervous system. Symptoms of early stage HAT are general and include intermittent fever, malaise, and lymphadenopathy. Infections caused by *T. b. gambiense* and *T. b. rhodesiense* differ primarily in the manifestation of the disease. The Gambian form of the disease is a chronic disease which takes years to progress, while Rhodesian trypanosomiasis is an acute infection in which the late stage develops only a few weeks or months following infection (26).

Four drugs are currently available for treatment of HAT, all of which are associated with problems, including toxicity, increasing incidences of treatment failure and the route of administration (14, 15). Pentamidine and suramin are used for the treatment of early stage trypanosomiasis. Pentamidine is used as a first line treatment for infections caused by *T. b. gambiense*, and suramin is used to treat *T. b. rhodesiense* infections (11, 15). Melarsoprol and eflornithine are used for CNS or second stage infections. Melarsoprol is the drug most often used to treat second stage CNS infections caused by both parasites. However, melarsoprol is associated with increasing reports of treatment failure (up to 30% in certain areas of central Africa), and it causes a reactive encephalopathy which can be fatal in up to 10% of treated patients (15). Eflornithine, also known as DFMO is only used against *T. b. gambiense* CNS infections (15). Additionally, all of the treatments currently used must be

administered by parenteral injection, which is difficult in the rural areas affected by trypanosomiasis (14). Thus, the need for new treatments is very pressing due to the problems associated with current therapy.

DB75 (2,5-bis(4-amidinophenyl)furan), also known as furamidine (Figure 2.1) is the active metabolite of DB289 (2,5-bis(4-amidinophenyl)furan-bis-*O*-methylamidoxime), a prodrug designed to orally treat HAT (*T. b. gambiense* is the parasite causing the current epidemic). DB289 recently began Phase III clinical trials in the Democratic Republic of Congo and Angola, and the trial has started recruiting patients in southern Sudan. DB75 is a diamidine analogue of pentamidine in which the alkoxy chain linking the phenyl rings has been replaced with a furan ring. DB75 has shown excellent in vitro and in vivo activity in mouse and monkey models of first stage infection (4, 7, 11, 13). DB820 (6-[5-(4-phenylamidinophenyl)-furanyl-2-yl]-nicotinamide), is an aza analogue of DB75. DB820 (Figure 1) is more potent than DB75 in the *T. b. rhodesiense* STIB 900 model of first stage infection, despite having similar in vitro activity against that strain (13). DB844, the O-methylamidoxime prodrug of DB820, has excellent activity in mouse and monkey models of CNS trypanosomiasis. DB844 is a leading candidate for the development of a new oral treatment for CNS trypanosomiasis.

Previous studies have shown that pentamidine is actively transported into trypanosomes via the P2, HAPT1 and LAPT1 transporters, with very high concentrations accumulating within trypanosomes (6, 8). Another diamidine, diaminazene, has been shown to be transported into trypanosomes primarily by the P2 transporter (9). Little is known, however, about the intracellular distribution of pentamidine, particularly over the time periods required for this slowly acting compound to kill trypanosomes. The lack of

fluorescence of pentamidine prevents microscopy studies which could determine localization. The structural modifications of DB75 and DB820 have increased the fluorescence emission of these compounds upon exposure to UV light. Therefore, we are currently able to combine studies of accumulation with studies of distribution in trypanosomes.

The mechanism of action of DB75 and DB820, as well as many other diamidines, is not known, and using fluorescence microscopy we can evaluate distribution to identify sites of action, or potential sites of loss, for the compounds in trypanosomes. In this study, a *T. b. brucei* strain, S427, was used to investigate the intracellular accumulation and intracellular distribution of the UV-fluorescent DB75 and DB820 using HPLC analysis and fluorescence microscopy.

This study determined that both DB75 and DB820 achieve millimolar concentrations in trypanosomes at each respective T_{max}, or the time at which the maximum concentration is achieved. DB75 and DB820 also demonstrate a progression in their intracellular distribution. These compounds first accumulate in the nucleus and kinetoplast, the DNA containing organelles of trypanosomes. By later time points, both are also found in organelles that are believed to be the acidocalcisomes as well as other non DNA-containing organelles. Accumulation at multiple sites in trypanosomes may be crucial to understanding the mechanism of action of DB75, DB820, and that of other diamidines.

C. Materials and Methods

Materials. Complete Baltz's Modified Essential Media (CBMEM) was prepared from Minimal Essential Media (MEM) supplemented with 100 mM hypoxanthine, 2 mM adenosine, 10 mM thymidine, 2 mM sodium pyruvate, 2 mM L-glutamine, 2-mercaptoethanol, and 10% v/v heat inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA). HPLC grade water and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA). All chemicals were obtained from Sigma – Aldrich Co (St. Louis, MO) unless otherwise specified.

Parasites. *T. b. brucei* strain S427 was cultivated in CBMEM media as described previously (2). Trypanosomes were maintained in Corning 24-well plates (Fisher Scientific) and were subpassaged every 3-5 days when trypanosomes reached a density of 10^5 – 10^6 trypanosomes per ml.

Anti-trypanosomal Agents. DB75 dihydrochloride salt was synthesized by Medichem (Chicago, IL) using previously described methods (5, 7). DB820 trihydrochloride salt was synthesized in the laboratory of Dr. David Boykin as previously described (13). For in vitro testing, DB75 and DB820 were prepared as 1 mM stocks in sterile water, and for in vivo testing the compounds were prepared in sterile saline (0.9% weight/vol).

In vitro Anti-trypanosomal Activity. In order to determine IC_{50} values, S427 trypanosomes (2×10^3 per ml) were cultured with serial dilutions of DB75 or DB820 for 70 h using a method described by Raz et al. (20). Alamar blue™ (10% vol/vol) (Biogen Intl, Camarillo, CA) was then added, and trypanosomes were further incubated for 2 h. The fluorescence of Alamar Blue was measured using a PolarStar fluorescence plate reader (BMG, Durham, NC) using an excitation wavelength of 544 nm and an emission wavelength

of 588 nm. Fluorescence emission at this wavelength is an indication of cell viability (20). The fluorescence emission derived from each drug concentration was normalized to fluorescence of the untreated control trypanosomes. The background fluorescence (determined in wells in which Alamar Blue (10% vol/vol) was added to CBMEM media) was subtracted from total fluorescence values. Dose response curves were generated from the Alamar Blue fluorescence shift using Prism™ (Graphpad Software, San Diego, CA). Experiments were performed in triplicate. IC_{50} values were determined using the Hill Equation.

In vitro accumulation in trypanosomes. To determine in vitro accumulation, 10^6 trypanosomes were incubated with either 7.5 μM DB75 or 7.5 μM DB820 for time periods ranging from 1 h to 24 h. At each time point, cells were washed twice in fresh drug-free media, lysed, and extracted in 4 volumes of 8:1 methanol: 0.1 N HCl (vol/vol). Half of each sample was evaporated in a Zymark (Hopkinton, MA) Turbovap-LV under nitrogen gas (7 psi) and reconstituted in 50 μl of HPLC solvent A (described in detail in **HPLC Analysis of DB75 and DB820**). Concentrations were determined as described below.

In vivo Anti-Trypanosomal Activity. Dose escalation studies in infected mice were used to determine in vivo anti-trypanosomal activity. All animal experiments adhered to guidelines outlined by the University of North Carolina-Chapel Hill Institutional Animal Care and Use Committee (IACUC). Each test group consisted of 6 mice obtained from Charles Rivers (Wilmington, MA). Male Swiss Webster (20-30 g) mice were inoculated with 10^5 S427 trypanosomes intraperitoneally on Day 0. On day three, mice were treated with a single intravenous dose (ranging from 0.63 to 10 μmol/kg) of either compound. The compounds were administered in 0.9% (weight/vol) saline. The dose volume administered to each

mouse was less than 0.2 ml. Untreated mice were administered the same saline vehicle. A drop of tail vein blood from each mouse was collected and examined for trypanosomes using a light microscope (40X magnification) daily for 2 weeks post-treatment. After that, tail vein blood was examined twice a week for 60 days. Mice were considered cured when no parasites were found in blood after the 60 day period. Untreated mice typically died within 5 to 6 days of infection. The percentage of animals cured in each test group was used to determine the dose response curve. ED_{50} values were calculated in Prism™ using the Hill Equation.

Plasma Concentrations of DB75 and DB820 in infected mice. Plasma concentrations of DB75 and DB820 were determined in infected animals. In these experiments, mice were treated with intravenous (IV) 7.5 μmol/kg of DB75 or DB820 on the third day after infection. Parasitemia was typically about $10^7 – 10^8$ trypanosomes per ml. Animals with lower parasitemia were excluded from the experiments before treatment.

Plasma samples were prepared as described previously (24), with the following modifications. Mice were euthanized by CO_2 asphyxiation, blood was collected by cardiac puncture, and the blood was separated into two lithium heparin-coated BD Microtainer tubes (Fisher Scientific), each with about 400-500 μl of blood. One microtainer tube of the blood was used for determining DB75 and DB820 plasma concentrations after a 7.5 μmol/kg dose IV through the tail vein, and the second was used to determine concentrations of DB75 and DB820 in trypanosomes isolated from the infected mice in certain experiments.

Blood was centrifuged at 3000 rpm (1500g) in a Marathon 8K centrifuge (Fisher Scientific) for 5 minutes to separate plasma from red blood cells. Plasma was removed, ensuring that the buffy coat was undisturbed. The buffy coat contains white blood cells and

trypanosomes in infected animals. Approximately 5 – 10 µl of plasma was examined under a light microscope (40 X - 100 X magnification) to ensure that it was not contaminated with trypanosomes. DB75 and DB820 were extracted from plasma in 4 volumes of methanol: 0.1N HCl (8:1 vol/vol), vortexed briefly and centrifuged at 3000 rpm (1500g) for 5 minutes. 100 µl of supernatant was removed and evaporated as described previously (24). The residue was reconstituted in 50 µl of HPLC solvent A (for each respective compound) and transferred to 200 µl polypropylene inserts for use in 2.0-ml glass HPLC vials (Agilent Technologies, Palo Alto, CA). Samples were analyzed by HPLC as described below.

Area Under the Curve (AUC) and half life ($t_{1/2}$) were determined from plasma concentrations using noncompartmental analysis in Winonlin 4.1 (Pharsight, Mountain View, CA). The $t_{1/2}$ was calculated from the terminal elimination rate constant (k_{el}), calculated between 4 and 24 hours. AUC was not extrapolated beyond 24 hours as concentrations in plasma were often below the limit of quantification after this time (21).

In vivo Accumulation of DB75 and DB820 in trypanosomes. Male Swiss Webster mice were infected with 10^5 trypanosomes per ml intraperitoneally. On the third day post infection, mice were treated with DB75 or DB820 at a dose level of 7.5 µmol/kg by a single tail vein injection. This dose level is curative for both compounds in this model of infection. At various time points after treatment, mice were euthanized by CO_2 inhalation, and blood was collected by cardiac puncture. The trypanosomes were collected by centrifuging the blood on a Percoll (Sigma) gradient as described by Grab and Bwayo (12), with minor modifications to the centrifugation conditions. Briefly, blood was mixed with an equal volume of 100% Percoll supplemented with 0.25 M sucrose and 0.11 M glucose, in 2.0-ml centrifuge tubes. The blood and Percoll mixture was centrifuged for 16100 g for 35 minutes

in an Eppendorf 5415R microcentrifuge (Fisher Scientific). Trypanosomes were removed from the supernatant, and washed twice (1 ml buffer) and counted. Compounds were extracted from the trypanosomes using 4 volumes of methanol: 0.1N HCl (8:1 vol/vol). Samples were evaporated under nitrogen gas (7psi) in a Zymark Turbovap and resuspended in 100µl of the HPLC solvent A for each respective compound. The average concentration of DB75 and DB820 in trypanosomes was determined using an estimated cell volume of 58 μm^3 (or 58 fl) for the S427 trypanosome (17). $AUC_{0\rightarrow 24h}$ and $t_{1/2}$ were calculated from trypanosome concentrations using noncompartmental analysis in Winonlin 4.1. As with plasma, $t_{1/2}$ was calculated from the k_{el} between 4 and 24 h.

Intracellular Localization of DB75 and DB820 in Trypanosomes. To determine the localization of DB75 and DB820 in trypanosomes, blood smears were made using a drop of tail blood at each time point after treatment with DB75 or DB820. Blood smears prepared from untreated mice were also examined. Blood smears were examined using Nikon Microphot FXA (Garden City, NJ) with a 60 DM x 1.4 NA objective lens, a mercury lamp, and an Optronics DEI 750 CCD camera (Goleta, CA). The microscope was equipped with a Nikon UV2A cube that limits excitation wavelengths to 330-380 nm and emission wavelengths to $\geq$ 420 nm.

HPLC Analysis of DB75 and DB820. Analytical methods for DB75 were conducted as described previously (24). Briefly the solvent system for DB75 analysis consisted of solvent A (15 mM ammonium formate/ 30 mM formic acid in 100% HPLC grade water) and solvent B had the same components in 4:1 acetonitrile: HPLC grade water. A gradient elution was used to resolve DB75, with a starting concentration of 88% solvent A:12% solvent B. This

gradient increased linearly to 80% solvent B over 40 min, followed by an 8 minute re-equilibration time to initial solvent conditions.

Analytical methods for DB820 were slightly different. For DB820, Solvent A contained 30 mM ammonium formate/60 mM formic acid in 100% HPLC grade water, and Solvent B was the same buffer in 4:1 acetonitrile: HPLC grade water. A gradient elution was used to resolve the compound, with a starting concentration of 94% Solvent A:6% Solvent B. This gradient increased linearly to 80% Solvent B over 40 min, followed by re-equilibration back to starting concentrations by 48 min.

An Agilent 1100 series HPLC equipped with a fluorescence detector (Agilent Technologies) was used for analytical procedures. DB75 and DB820 were eluted on a 5.0 μm Bonus – RP (Agilent Technologies) 2.1 x 150 mm column with a flow rate of 0.35 ml/min. The column was maintained at 40 ºC throughout the analytical method. Injection volume for each sample was 25 μl. The wavelengths used for fluorescence detection were λ_{ex} = 365 nm and λ_{em} = 462 nm. Compounds were quantified by comparing to standards injected during each analytical run. This was used to determine the concentration of DB75 and DB820 in plasma and in trypanosomes. Concentrations for both compounds were expressed as μM and mM for plasma and trypanosomes, respectively. Concentrations are reported as plus or minus the SE.

D. Results

In vitro activity of DB75 and DB820. Both DB75 and DB820 had potent activity against the *T. b. brucei* S427 strain. The IC_{50} values were less than 10 nM (Table 2.1). The related compound, pentamidine, was slightly more potent than DB75 and DB820 in vitro with an IC_{50} value of 1.1 nM.

In vitro accumulation of DB75 and DB820. Trypanosomes growing in vitro were exposed to DB75 or DB820 at concentrations of 7.5 μM, approximately 1000 times the IC_{50} values. This concentration is similar to that used in previous studies with pentamidine (5). Uptake of both DB75 and DB820 increased over time, with millimolar concentrations in trypanosomes at 24 h (Figure 2.2). There appears to be two rises in accumulation for both compounds – after an initial rise in concentrations, there is a slight plateau before concentrations rise again. Although DB75 and its aza analogue had similar in vitro activity, DB75 accumulated to a greater extent in trypanosomes than DB820 over the 24 h period. At 24 h, trypanosomes accumulated DB75 to a concentration of 12.2 ± 0.8 mM (± SE), or 70.8 ± 4.7 nmol/10^8 trypanosomes, almost five times the concentration of DB820 in trypanosomes at that time (2.6 ± 0.2 mM, or 15.0 ±1.4 nmol/10^8 trypanosomes). Thus, DB820 and DB75 were concentrated inside trypanosomes approximately 400-2000 fold the extracellular incubation concentration at 24 h.

In vivo activity of DB75 and DB820. In vivo efficacy experiments showed that DB75 and DB820 were both very potent in vivo, with comparable dose response effects in terms of cure rates and survival times (Table 2.1). A single IV dose of either DB75 or DB820 at 10 μmol/kg cured all mice infected with *T. b. brucei* S427. Cure was defined as survival with no parasitemia for 60 days after treatment. ED_{50} values calculated from the cure rates were

comparable (1.3 μmol/kg for DB75 and 1.6 μmol/kg for DB820). Pentamidine was less active in vivo, failing to completely cure all animals at 10 μmol/kg IV. The extrapolated ED_{50} value for pentamidine was 3.2 μmol/kg. This ED_{50} value is higher than previously reported for pentamidine against the S427 strain, which was between 0.9 and 1.0 μmol/kg (or 0.5-0.6 mg/kg) (3). However in this experiment, mice were infected with trypanosomes, and treatment was begun 24 hours later. In the experiments reported here, treatment was begun 3 days post infection, which is a more stringent efficacy model. The values reported here are still very similar to those determined previously.

In vivo accumulation of DB75 and DB820 in trypanosomes and plasma. Mice infected with *T. b. brucei* S427 were dosed with DB75 or DB820 at 7.5 μmol/kg IV. Mouse plasma concentrations and trypanosome intracellular concentrations for both compounds were measured over a 24 hr period (Figure 2.3).

Plasma concentrations of DB75 and DB820 in infected animals. The plasma time concentration profiles for DB75 and DB820 in infected mice were similar (Figure 2. 3). The peak plasma concentration of DB75 in infected mice after IV administration was 295 ± 93 nM at 15 min post treatment (Figure 3A), the earliest time point measured. The Area Under the Curve between 0 and 24 hours ($AUC_{0\rightarrow 24h}$) of DB75 in the plasma infected mice was 1.8 μM*h. The plasma half-life ($t_{1/2}$) of DB75 was 4.5 h. The peak concentration of DB820 in plasma was 442 ±145 nM at 15 min post treatment (Figure 2.3B). The AUC for DB820 in infected plasma was 1.9 μM*h, with $t_{1/2}$ of 12.4 h. The half lives for DB75 and DB820 in plasma were calculated between 4 and 24 h.

In vivo accumulation of DB75 and DB820 in trypanosomes. To determine concentrations of DB75 and DB820 in trypanosomes, trypanosomes were isolated from

blood at multiple time points after the dosing. Concentrations of DB75 and DB820 that accumulated in trypanosomes after a single 7.5 μmol/kg dose are plotted in Figure 2.3. Both compounds were rapidly taken up and concentrated within trypanosomes, with 0.5 mM intracellular concentrations at 15 min post treatment, the earliest time point measured. DB75 peaked in trypanosomes at a concentration of 0.9 ± 0.2 mM (5.4 ± 1 nmol/10^8 trypanosomes) at 4 h after administration, approximately 6,000 times the corresponding plasma concentration. The $AUC_{0\rightarrow 24h}$ for DB75 in trypanosomes was approximately 9,400 μM*h. The concentration of DB820 peaked in trypanosomes slightly later, at 4 h, with a maximum concentration of 1.8 ± 0.7 mM (9.6 ± 4.3 nmol/10^8 trypanosomes), which is about 17,000 times the concentration in plasma at 4 h. The $AUC_{0\rightarrow 24h}$ for DB820 in trypanosomes was approximately 17,000 μM*h. Both DB75 and DB820 concentrations in trypanosomes declined over time with half-lives of 4.9 and 6.4 h respectively.

Intracellular distribution of DB75 and DB820 in trypanosomes. Fluorescence microscopy was used to determine the intracellular distribution of DB75 and DB820 within organelles of trypanosomes. The fluorescence micrographs (Figure 2.4) show that DB75 and DB820 rapidly accumulated in trypanosomes, concentrating initially in the DNA-containing nucleus and kinetoplast. At 15 min, when intracellular concentrations of DB75 and DB820 were approximately 0.5 mM, cell nuclei showed a bright blue fluorescence. The kinetoplast was also clearly visible at this early time point, with very intense white-yellow fluorescence observed (Figure 2.4A and 2.4F). Nuclear and kinetoplast fluorescence is apparent at all time points examined to some degree, but the kinetoplast is not always yellow, as seen in Figure 2.4E. Occasionally, some of the trypanosomes depicted at 24 h in the micrographs do not have fluorescent kinetoplasts (Figure 2.4J). This appears to be a true phenomenon, with

approximately 1/4 of trypanosomes in multiple fields showing no kinetoplast fluorescence at 24 h.

Additionally, DB75 appears in unidentified punctate organelles in the perinuclear region and at the tip of the trypanosome where the flagellum emerges as a yellow fluorescence (Figure 2.4A). This also occurs with DB820 (Figure 2.4F), but not as much as with DB75. As early as 2 h post treatment, both compounds are found in organelles believed to be the acidocalcisomes based on a similar fluorescence pattern as acridine orange in trypanosomes (10). DB75 appears as a yellowish color in these organelles, while DB820 is a yellowish-orange color. The DB75 and DB820 accumulating organelles are usually concentrated at the posterior end of the trypanosome. By 4 hours, the number of yellow fluorescent organelles increases (Figure 2.4E and 2.4J), and the numbers remain that way through 24 hours. It is interesting to note that after 4 hours, concentrations of both compounds in trypanosomes begin declining.

E. Discussion

DB75 and DB820 are potent analogues of pentamidine. Although all three have very similar in vitro activity, in vivo, it is clear that both DB75 and DB820 have better anti-trypanosomal activity than pentamidine. Even at the highest dose tested, 20 μmol/kg IV of pentamidine, we were unable to cure the infection in all animals (unpublished results). Additionally, in the more stringent model of first stage trypanosomiasis, the STIB 900 *T. b. rhodesiense* model, DB820 is the most potent of these three compounds (13). Both DB75 and DB820 can also be administered as lipophilic prodrugs, which enables an oral formulation for the treatment of trypanosomiasis (24). This is useful, especially for early stage disease, because it eliminates the need for parenteral administration. The prodrug of DB75, DB289, is currently in Phase III clinical trials for sleeping sickness. DB844, the prodrug of DB820, is an excellent drug candidate for the treatment of CNS trypanosomiasis. DB844 is able to cure mouse models infected with the chronic GVR35 strain of *T. b. brucei* after an oral dose of 5 × 100 mg/kg, and is being investigated in further animal models.

In these studies, *T. b. brucei* accumulates millimolar concentrations of both DB75 and DB820 after in both vitro incubation and after in vivo administration to infected mice (Figure 2.2 and Figure 2.3). The in vitro concentrations are very similar to concentrations previously reported for pentamidine accumulation published by Carter et al. (6). In vitro, they found that pentamidine was accumulated in 10^8 cells to a concentration of 1 mM, or 6.05 nmol/10^8 cells, after a 3-hour in vitro incubation with 1 μM pentamidine. This concentration of pentamidine is approximately 1000 fold the IC_{50} value. In the experiments presented here, trypanosomes were incubated with 7.5 μM of DB75 or DB820, which was approximately 1000 times the IC_{50} value for both compounds, and both accumulated at millimolar levels in

vitro over a 24-h period (Figure 2.2). After 4 hours, DB75 and DB820 are concentrated in trypanosomes almost 200 times the initial concentration, whereas in Carter's experiments, pentamidine reaches levels inside trypanosomes that are approximately 1000 times the incubation concentration.

The in vitro accumulation differences of pentamidine and DB75 and DB820 could be explained by the differing mechanisms for transport into trypanosomes. Pentamidine is transported into trypanosomes by three transporters, the high and low affinity pentamidine transporters (HAPT1 and LAPT1) and the P2 transporter (8). The P2 transporter has also been shown to accumulate DB75 and related diamidines (23), but to our knowledge, there is no evidence that HAPT1 or LAPT1 can transport DB75 and DB820 into trypanosomes. Therefore, pentamidine could have accumulated to a greater extent in trypanosomes due to the multiple routes of drug entry, which may not be available to DB75 and DB820. A more accurate comparison for DB75 and DB820 and related diamidines may be diaminazene, which is taken up into trypanosomes almost exclusively through the P2 transporter (9).

DB75 and DB820 were shown to accumulate in trypanosomes to concentrations of 1-2 millimolar levels after in vivo treatment (Figure 2.3). DB820 has a higher peak concentration and higher $AUC_{0\text{-}24h}$ in trypanosomes than DB75. The same holds in plasma – DB820 had a higher peak concentration and slightly higher $AUC_{0\text{-}24h}$ in infected plasma than DB75. For most time points, DB820 concentrations in infected plasma were higher than DB75 plasma concentrations. Plasma concentrations were also variable, which could be a result of the infection itself. At the peak concentration in trypanosomes, the organisms were able to accumulate between 6,000 and 17,000 times the plasma concentrations, which is a very large concentration factor.

Previously published data (6) suggests that the concentration of pentamidine in vivo in trypanosomes after a 4 mg/kg dose administered intraperitoneally to rats is approximately 0.84 mM, lower than what was shown here for DB75 and DB820. A 4 mg/kg dose of pentamidine is equivalent to approximately 6.75 μmol/kg of the isethionate salt, and therefore the dose of pentamidine administered by Carter et al. was slightly lower than the dose of DB75 and DB820 administered in our studies, 7.5 μmol/kg. Also the route of administration used for pentamidine in those experiments was intraperitoneal injection. Thus, pentamidine required additional time to enter circulation and distribute into trypanosomes, and the peak concentration may not have occurred at their tested time point of 4 hours. DB75 and DB820 were administered by intravenous injection, and do not require extra time to distribute into circulation. Both the lower dosage and the different route of administration could explain the lower concentration of pentamidine in trypanosomes at 4 hours. It is also our experience that diamidines need to be administered at a much higher dose intraperitoneally to achieve comparable cure rates to a dose after intravenous administration. Although the routes of administration are different in the two studies and there are other confounding factors, it is clear that trypanosomes are able to accumulate high concentrations of diamidines which leads to killing of the organisms.

Diamidines such as DB75, DB820, and pentamidine are able to bind DNA in AT rich regions of the minor groove (5, 28). In fact, one of the long hypothesized mechanisms of action has been binding to DNA and/or interference with DNA associated enzymes such as Topoisomerase II (21, 22). We expected to, and did, see accumulation of DB75 and DB820 in the nucleus and the kinetoplast of trypanosomes, the two DNA containing organelles. Although DB75 and DB820 bind to DNA in both the nucleus and the kinetoplast, it is not

known for certain what effects this may have, or whether accumulation in one organelle has a greater impact on the death of the trypanosome. Future studies are planned to investigate whether different amounts of compound bind to the DNA in these organelles and how this affects the mechanism of action of the compounds.

Accumulation in other organelles, such as the organelles believed to be acidocalcisomes and several unknown perinuclear organelles was unexpected. Accumulation in these organelles begins about 1 hour after treatment is administered. This organelle accumulation could represent some unknown mechanism of action for diamidines, or it could be a source of loss for the compounds. If these organelles are acidocalcisomes, it is possible that the diamidines may have some kind of interaction with polyphosphates that are sequestered there. Acidocalcisomes have been postulated to be involved in many processes, including pH regulation and osmoregulation within trypanosomes. Disruption of these processes could potentially be a mechanism of action. Additionally, these diamidines could interfere in polyphosphate accumulation and hydrolysis in acidocalcisomes, a mechanism that has previously been shown to interfere with trypanosome virulence in animals (16). Supporting the idea of accumulation in the acidocalcisomes as a mechanism of action of these compounds, is the investigation by Ormerod and Shaw (19). In this study it was found that stilbamidine and hydroxystilbamidine accumulate in "volutin granules" in trypanosomes. One of these authors also noted a shift in the fluorescence of hydroxystilbamidine in these granules, much as is seen here with DB75 and DB820 (18). It is important to consider that there may be more than one mechanism of action for diamidine compounds as a class. Additionally, individual diamidines may display one or more of these mechanisms of action.

In future studies we hope to examine the mechanism of action for these and other diamidine compounds.

Another question that arises from the distribution micrographs shown in Figure 3 is what could explain the shift in fluorescence seen with DB75 and DB820 in the kinetoplast, acidocalcisomes, and other organelles. DAPI has been postulated to undergo a red shift in its fluorescence emission when accumulated in volutin granules of yeast (1). Volutin granules or the acidocalcisomes in trypanosomes concentrate polyphosphates and pyrophosphates at high concentrations, and it has been hypothesized that diamidines such as DAPI interact with phosphates, leading to a shift in the fluorescence emission (25). It appears that the same phenomenon occurs with DB75 and DB820 in the posterior organelles, but this does not explain why there is a shift in the fluorescence emission in the kinetoplast. Potentially, two different mechanisms could cause the shifts in fluorescence in the organelles. Fluorescence scans of DB75 solutions prepared at low pH indicated no shift in the fluorescence spectrum to longer wavelengths; therefore the shift that occurs in these organelles probably does not occur due to low pH found inside any of the organelles (J. E. Hall, unpublished data).

The studies described here have opened many opportunities for the further study of diamidine compounds. The use of fluorescence to track the distribution of compounds in trypanosomes will enable us to examine uptake and intracellular accumulation as well as distribution and mechanism of action. When combined with HPLC analysis of concentrations of drugs in trypanosomes, this is an exciting technique to evaluate drugs and their anti-trypanosomal activity. The studies described here will be extended to other compounds in a library of almost 2000 structurally diverse diamidines in order to assess distribution and accumulation over time and how that relates to activity. Furthermore, using

techniques to isolate trypanosome organelles, it will be possible to evaluate the accumulation of the compounds in each organelle in order to determine how that may relate to mechanism of action.

F. Acknowledgements

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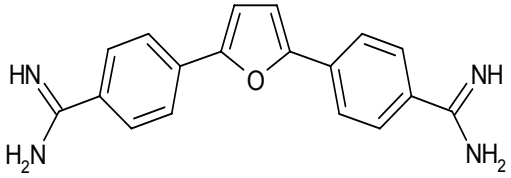
Table 2.1. in vitro and in vivo activity of diamidine compounds

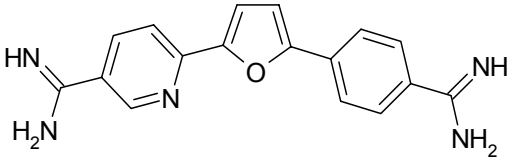
Compound	In vitro IC_{50} (nM)	Dose (μmol/kg)	Cure Rate (%)	Mean Survival Time (days)[a]
Pentamidine	1.1	1 x 10	67	51
		1 x 5	67	50
		1 x 1.25	0	10
		1 x 0.63	0	7
DB75	3.4	1 x 10	100	60
		1 x 5	67	47
		1 x 1.25	36	37
		1 x 0.63	17	24
DB820	7.3	1 x 10	100	60
		1 X 7.5	83	58
		1 x 5	75	49
		1 x 1.25	25	35
		1 x 0.63	0	9

[a] Mean Survival Time for untreated control animals was 5-6 days.

Figure 2.1. Chemical structures of DB75, also known as furamidine, and DB820, anti-trypanosomal diamidine derivatives of pentamidine.



DB75 (Furamidine)



DB820

Figure 2.2. In vitro accumulation of DB75 (▲) and DB820 (■) over 24 hours (n=3). Trypanosomes were incubated with 7.5 μM of either compound. Concentrations are presented ± SE.

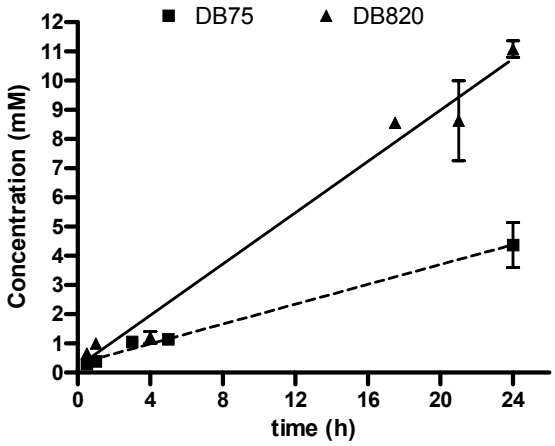


Figure 2.3. Concentration of DB75 (A) and DB820 (B) in S427 trypanosomes and plasma after intravenous administration of 7.5 μmol/kg of DB75 or DB820. Experiments were performed with 3-6 mice per time point. Concentrations in trypanosomes were determined based on a volume determination of 58μm^3, determined by Opperdoes et al (16). Concentrations are presented ± SE.

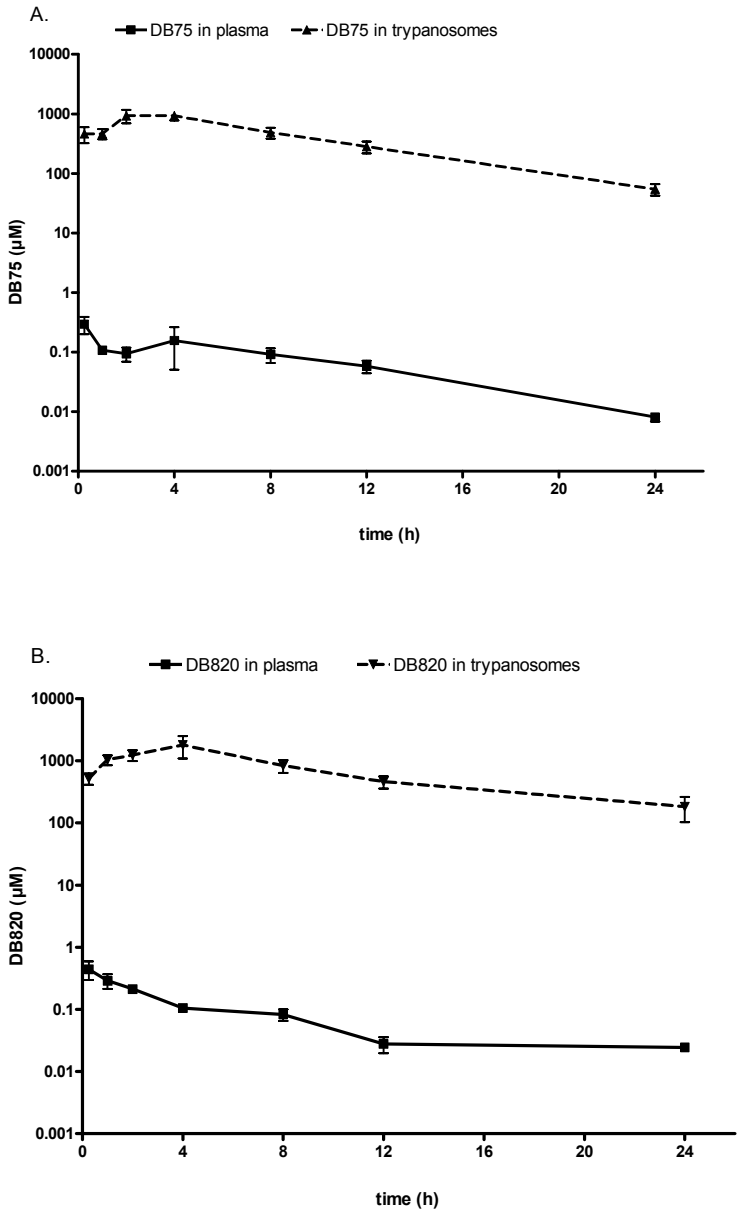
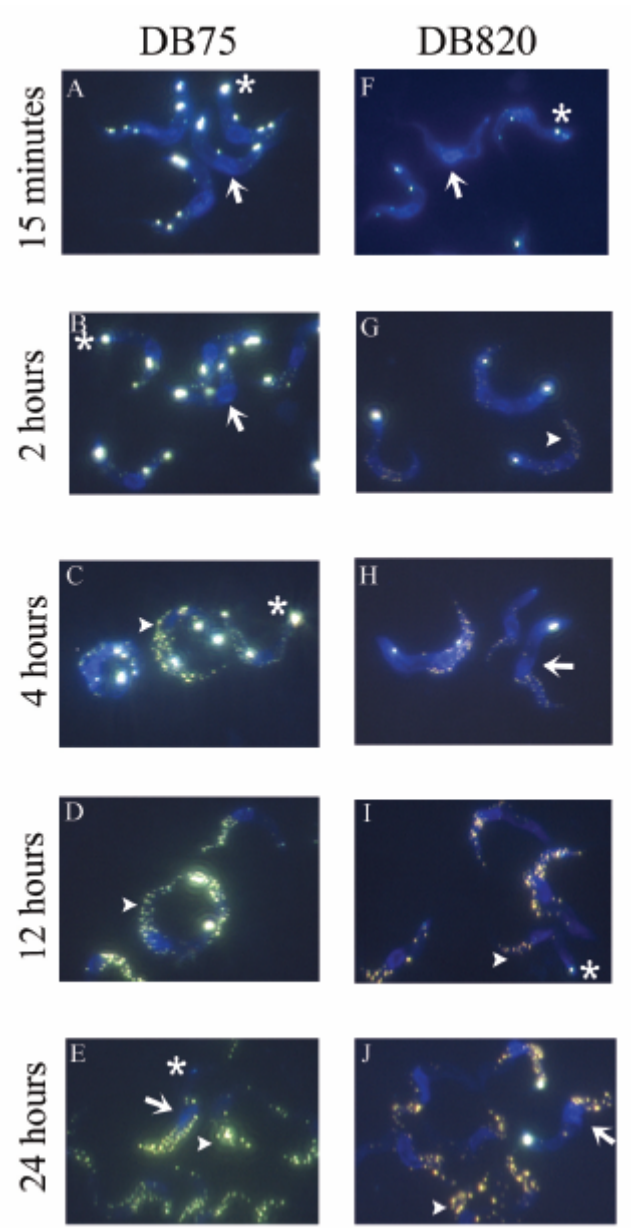


Figure 2.4. Fluorescence micrographs of DB75 and DB820 in trypanosomes after 7.5 μmol/kg intravenous dose of either compound. A-E show selected time points from DB75 treated mice, while F-J are from DB820 treated mice. In select micrographs, the nucleus is represented by the arrow (→), the kinetoplast is depicted by an asterisk (*), and the acidocalcisomes are indicated by an arrowhead (►).



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CHAPTER 3

ACCUMULATION AND DISTRIBUTION OF DB75 IN TRYPANOSOMES LACKING THE P2 TRANSPORTER

A. Abstract

DB75, or furamidine, is the active diamidine of DB289, currently in Phase III clinical trials in Africa for the treatment of early stage human African trypanosomiasis. DB75 has been shown in the past to accumulate in trypanosomes via the P2 transporter on the trypanosome cell surface. Accumulation and intracellular distribution of DB75 in three lines which lack the drug uptake transporter activity was investigated. These diamidine resistant lines were approximately 20 fold resistant to DB75 in vitro. DB75 accumulated to high concentrations in trypanosomes over time both in vitro and in vivo, although to a lesser extent in the resistant lines than in the wild type S427 line. DB75 accumulated in vitro to concentrations between 30 – 60 % of concentrations seen in the wild type line in all three resistant lines, *TbAT1-/-*, DB75^{R}-CL1, and B48. Accumulation in the three resistant lines was greatly reduced in vivo in comparison to the wild type line, especially at the low dose of 5 μmol/kg. There was a lack of dose proportionality seen with both trypanosome and plasma concentrations. Trypanosome exposure in the resistant lines was only 5-20% of that seen in the wild type S427 line at both doses administered. Additionally, intracellular distribution studies showed that the spread of DB75 to organelles in the resistant lines was much slower than that seen in the wild type S427 trypanosomes. In vivo, in the resistant lines, DB75 did not localize extensively to the acidocalcisomes, and was primarily found in the kinetoplast and nucleus. Accumulation in the resistant lines, while not as extensive as in the wild type line, indicated that there may be alternate routes of drug accumulation in trypanosomes.

B. Introduction

Human African trypanosomiasis, a parasitic infection caused by *Trypanosoma brucei* spp., is found in sub-Saharan Africa. Trypanosomiasis has the potential to affect millions of people in this African region (19). Treatments available are limited primarily to four drugs which were developed, for the most part, many years ago. The diamidine pentamidine and polysulfated naphthalene suramin are used to treat trypanosomiasis infections in the early, or hemolymphatic stage, while melarsoprol and eflornithine are treatments used for the late stage of infection, or when the parasite has infected the central nervous system (CNS) (15). The prodrug pafuramidine, also known as DB289, is currently in phase III clinical trials for the treatment of early stage trypanosomiasis.

Diamidines, as positively charged molecules at physiological pH, should have slow rates of passive diffusion across cellular membranes (4). DB75 has been shown to have limited permeability across Caco2 cell monolayers, especially in relation to its prodrug DB289 (20). Several transporters on the surface of trypanosomes have been implicated in the uptake of diamidines in trypanosomes. Activity for a high affinity and low affinity transporter for pentamidine (HAPT1 and LAPT1) has been determined (4), but no endogenous substrates for these transporters have been identified (5). A purine nucleoside transporter, the P2 transporter, has also been found to transport pentamidine, melarsoprol, diminazene, and DB75 into trypanosomes (1, 4, 5, 7, 14).

DB75 is the active diamidine formed from DB289, and has been shown to accumulate in trypanosomes through the P2 transporter to high concentrations (10, 11). Short term uptake experiments have shown that the P2 transporter is the primary means of DB75 accumulation in trypanosomes, and when the transporter activity is eliminated, accumulation

is minimal (10). Use of only one uptake transporter could lead to the development of drug resistant parasites in the future, which is important to consider during the drug development process. Pentamidine, a widely studied diamidine, has been shown to accumulate in trypanosomes by three uptake transporters, P2, HAPT1 and LAPT1, which could explain the lack of resistance associated with this compound despite wide use as a prophylactic for many years in Africa (15).

Melarsoprol and diaminazene (Berenil) are used for human and animal trypanosomiasis, respectively. Both compounds have been shown to be transported by the P2 transporter, but *TbAT1-/-* trypanosomes are 20-fold resistant to diaminazene in comparison to the wild type strain, while the trypanosomes are only 2-3 fold resistant to melarsoprol (14). Interestingly, several melarsoprol resistant trypanosome field isolates have mutated P2 transporters (13), indicating the importance of this transporter for melarsoprol uptake.

Several trypanosome lines have been developed with alterations to various uptake transporters. The *TbAT1-/-*, or P2KO, is a double knockout of the *TbAT1* allele, which encodes the gene for the P2 transporter (14). Additionally, a laboratory line has been generated which is resistant to DB75, and is designated $DB75^{R}$-CL1. Genetically, this line is also missing the DNA encoding the P2 transporter (10). A third line, B48, has been generated from the *TbAT1-/-*. To develop this line, *TbAT1-/-* was made pentamidine resistant, and has lost the activity of HAPT1 (3). All of these lines are resistant to DB75, and display reduced uptake of the compound and other diamidines over short periods of time. However, the impact of the lack of the P2 transporter over longer periods of time (i.e. hours) has not been investigated until now.

These investigations show that even in trypanosomes lacking the P2 transporter, accumulation of DB75 still occurs over time, although to a lesser extent than that of the wild type S427 line. The accumulation in the resistant lines lacking the P2 transporter may be indicative of other transport mechanisms able to accumulate DB75 in trypanosomes. Distribution studies also show that in resistant trypanosomes, intracellular distribution is altered. Decreased accumulation and altered distribution may explain lower in vitro and in vivo potency seen in these resistant trypanosome lines.

C. Materials and Methods

Trypanosomes. The S427 *Trypanosoma brucei brucei* line was used as a wild type control in all experiments. *TbAT1-/-* was derived from S427 as previously described (14). The DB75^{R}-CL1 was derived by incubating S427 trypanosomes with increasing DB75 pressure as previously described (10). B48 was the *TbAT1-/-* line further selected for pentamidine resistance (3). B48, in addition to loss of the P2 transporter, has lost HAPT1 activity. *TbAT1-/-* and DB75^{R}-CL1 were obtained from Dr. Mike Barrett, and B48 was obtained from Dr. Harry de Koning. The resistant lines were originally cultured in HMI-9 media, and were adapted to CBMEM media for in vitro culture. All four lines were then cultured in CBMEM media, as previously described (11).

In vitro Anti-trypanosomal Activity. To determine IC_{50} values, *T. b. brucei* S427 and *T. b. rhodesiense* STIB900 trypanosomes (2 x 10^4 per ml) were cultured at 37 ºC and 5% CO_2 with serial dilutions of DB75 for 72 h using a method previously described (16). After 70 h, 10 % vol/vol Alamar Blue was added to trypanosomes. Fluorescence intensity (ex: 544 nm, em: 588 nm) was measured 2 h later to determine viability of trypanosomes. Experiments were performed in triplicate with each trypanosome line. IC_{50} values were determined using the Hill Equation in Prism 4.0 (Graphpad, San Diego, CA) and are the average of at least 3 determinations. Fold resistance was calculated by dividing IC_{50} values determined for *TbAT1-/-*, DB75^{R}-CL1, and B48 lines by the IC_{50} value determined for the wild type S427 line.

In vitro Time-Dose Response. Time – dose experiments were performed using a method adapted from Kaminsky and Brun (8). These experiments were used to investigate how long trypanosomes need to be exposed to DB75 in vitro for the compound to kill the cells.

Briefly, trypanosomes were incubated with either 500 nM or 7.5 μM for periods of time between 1 h and 48 h (1h, 2h, 4h, 8h, 24h, 48h). At each time point, trypanosomes were washed twice in 1 ml of medium and resuspended in drug free medium. Trypanosomes were inspected daily for survival and motility. When all trypanosomes in a well were dead as determined by microscopic inspection, the time of death and corresponding incubation time of DB75 were recorded.

In vitro accumulation of DB75. The in vitro accumulation of DB75 was determined in all four lines over 8 h, as previously described (11). Briefly, 10^6 trypanosomes were incubated with DB75 for varying periods of time between 1 and 8 h. At each time point, trypanosomes were washed twice in drug free medium (1 ml), and DB75 was extracted from cells with 4 volumes of methanol: 0.1 N HCl (8:1 vol/vol). Half of each sample was evaporated in a Zymark (Hopkinton, MA) Turbovap-LV under nitrogen gas (7 psi) and reconstituted in High Performance Liquid Chromatography (HPLC) solvent A (described in detail in **HPLC Analysis of DB75**). The remainder of the sample was retained for future analysis if needed. DB75 concentrations in trypanosomes were determined as described previously (11). Accumulation of DB75 in each line was measured at 3 concentrations: 150 nM, 500 nM and 7.5 μM. 150 nM was chosen as it is approximately the IC_{50} value of the resistant lines. 500 nM and 7.5 μM were 6 fold lower than and 2 fold higher than the km of DB75 for the P2 transporter, respectively.

In vivo Accumulation of DB75 in Trypanosomes and Plasma. Male Swiss Webster mice (25-35 g) were infected with each line of trypanosomes (10^5 trypanosomes) intraperitoneally (IP). All animal experiments adhered to guidelines outlined by the University of North Carolina-Chapel Hill Institutional Animal Care and Use Committee (IACUC). On the third

day post infection, parasitemia was determined by microscopic examination of a drop of blood from the tail vein. Infected mice were treated with one of two doses of DB75 administered IP: 5 μmol/kg or 15 μmol/kg. At time points after treatment was administered, mice were euthanized by CO_2 asphyxiation, and blood was collected by cardiac puncture into two lithium heparin coated microtainer tubes (BD Biosciences). As previously described (11), one tube was used to isolate trypanosomes from whole blood, while the other was used to determine plasma concentrations of DB75. Briefly, trypanosomes were isolated by mixing whole blood with a Percoll solution (100%) containing 0.25 M sucrose and 0.1 M glucose (pH 7.4). This mixture was centrifuged for 35 min at 13200 rpm (16100 g) in an Eppendorf 5415R microcentrifuge. Trypanosomes were isolated from suspension above the red blood cell layer, and were washed twice in 1 ml of phosphate saline glucose (PSG), counted and drug was extracted with four volumes of 8:1 methanol: 0.1N HCl (vol/vol). Half of each sample was evaporated in a Zymark (Hopkinton, MA) Turbovap-LV under nitrogen gas (7 psi) and reconstituted in (HPLC) solvent A. The remainder of the sample was retained for future analysis if needed. Diamidine concentrations in trypanosomes were determined as described previously (11). Plasma was isolated by centrifuging blood at 3000 rpm (1500 g) for 10 min to pellet red blood cells, and extracted as described above.

Intracellular Distribution of DB75 in Trypanosomes. Intracellular distribution of DB75 in the wild type S427, *TbAT1-/-*, $DB75^R$-CL1, and B48 lines was compared over time in vitro and in vivo. For in vitro experiments, approximately 10^6 trypanosomes were incubated with 500 nM DB75. At time points ranging from 15 min to 8 h after the incubation was started, trypanosomes were removed, washed twice in drug free medium (1 ml), and then resuspended in 20 μl of freshly isolated mouse blood. A small drop of blood was placed on a

microscope slide and thin films were prepared. Thin films were viewed within 1 day of preparation. For in vivo experiments, thin films were prepared at each time point in above mentioned pharmacokinetic studies.

Fluorescence Microscopy of DB75 in Trypanosomes. Thin films were mounted with a drop of glycerol before observation. A Nikon Microphot FXA microscope (Garden City, NJ) with a 60 DM x 1.4 NA objective lens, a mercury lamp, and QImaging Micropublisher 3.3 CCD Digital Camera (QImaging Corporation, Surrey, BC, CA) was used for fluorescence microscopy. QCapture version 3.0 imaging software was used to capture images (QImaging). The microscope was equipped with a Nikon UV2A cube that limits excitation wavelengths to 330-380 nm and emission wavelengths to ≥ 420 nm. The UV2A cube allows for the visualization of the blue fluorescence seen in the nucleus and kinetoplast, and the yellow/orange fluorescence seen in the acidocalcisomes due to interactions of diamidines with polyphosphates in these organelles, as seen previously with yeast volutin granules (18).

HPLC Analysis of DB75 in Trypanosomes. HPLC analysis of DB75 was performed in a manner similar to that previously described (11). Evaporated samples were reconstituted in HPLC Solvent A (15 mM ammonium formate, 35 mM formic acid in 100% HPLC grade water). A gradient elution was used to resolve DB75, with a starting concentration of 95% Solvent A, 5% Solvent B (15 mM ammonium formate, 35 mM formic acid dissolved in 4:1 ratio of acetonitrile and HPLC grade water). Over 18 min, % B was linearly increased to 100%, followed by a 5 min post time re-equilibration to starting conditions.

An Agilent 1100 series HPLC equipped with a fluorescence detector (Agilent Technologies) was used for analytical procedures. DB75 was resolved on a 3.5 μm Zorbax

Bonus – RP 2.1 x 50 mm column (Agilent Technologies, New Castle, DE) with a flow rate of 0.35 ml/min. The column was maintained at 25 ºC throughout the analytical method. Injection volume for each sample was 5 µl. The wavelengths used for fluorescence detection were λ_{ex} = 365 nm and λ_{em} = 462 nm. Compounds were quantified by comparing to standards injected during each analytical run. The standard was used to determine the concentration of DB75 in plasma and in trypanosomes. Concentrations for both compounds were expressed as µM for plasma and trypanosomes (based on trypanosome volume of 58 μm^3, or 58 fl). Concentrations are reported as plus or minus the SE.

D. Results

In vitro Anti-trypanosomal Activity of DB75. The in vitro anti-trypanosomal activity of DB75 was determined in four *T. b. brucei* lines of trypanosomes – the S427 wild type, and three resistant clones derived from it, *TbAT1-/-*, $DB75^R$-CL1, and B48 (derived from *TbAT1-/-)*. DB75 was a potent compound against the wild type line S427 (Table 3.1), with an IC_{50} value of 7 nM. DB75 was less potent against the three resistant lines, which had approximately 30 fold resistance to DB75 in comparison to the wild type line. There was no significant difference in the IC_{50} values for the three resistant lines, but the activity of DB75 against the three resistant lines was significantly different from that seen in the wild type line (p value = 0.007, 0.03, and 0.03 for *TbAT1-/-,* $DB75^R$-CL1, and B48 respectively, as determined by Student's T-test). Removal of HAPT1 activity, which is known to be one of the transporters involved in uptake of pentamidine into trypanosomes (3), did not further increase resistance to DB75 in vitro.

In vitro Time-Dose Response of DB75. The incubation time with DB75 required to kill trypanosomes was determined. Each line of trypanosomes was incubated with either 500 nM or 7.5 μM DB75 for periods of time ranging between 1 h and 48 h. With the wild type S427, DB75 only needed to be exposed to trypanosomes for 1 h in order for death to occur (Table 3.2). Although only 1 hour exposure was sufficient to kill trypanosomes at either concentration, the actual time required for trypanosomes to die was at least 48 h, as DB75 is a slow acting compound. With the resistant lines, DB75 needed to be incubated with trypanosomes for much longer periods of time in order for killing to occur. Again, cell death did not occur for at least 48 h. At the 500 nM dose, DB75 needed to be incubated with the resistant trypanosomes for at least 24 h for death to occur at 48 h or a few hours later,

whereas at the higher dose, 7.5 μM, the resistant trypanosomes needed to be incubated with DB75 for between 4-24 h, depending on the line (Table 3.2). When a range of time is given, at least one of the three wells had live trypanosomes at the shorter incubation time, but all trypanosomes were dead after the longer exposure time. It is apparent that even when the P2 transporter is not present in trypanosomes, DB75 still accumulates sufficiently inside the cells to cause death, but either accumulation is less than in the wild type S427 or occurs by a much slower route, and therefore longer exposure times are needed to kill trypanosomes.

In vitro Accumulation of DB75 in Trypanosomes. Accumulation of DB75 in the wild type and resistant lines was measured at three incubation concentrations: 150 nM, 500 nM, and 7.5 μM (Fig 3.1). The concentrations were chosen for the following reasons: 150nM is the approximate IC_{50} value for DB75 against the resistant lines, and 500 nM and 7.5 μM are 6 fold lower and 2 fold higher than the Km value of DB75 for the P2 transporter (3.2 μM), as determined by Lanteri et al. (10). We also attempted to investigate the accumulation of DB75 after 10 nM incubation, but concentrations in trypanosomes were at or below the limit of quantification by LC/MS detection.

In vitro accumulation after exposure to 150 nM DB75. In the wild type S427 line, DB75 was accumulated to a peak concentration of approximately 300 μM at 8 h (Figure 3.1A), and concentrations of DB75 increased over all time points measured. In comparison, accumulation in the three resistant lines was lower and did not increase substantially over time. After 8 h incubation the *TbAT1-/-* the DB75 concentration was approximately 50 μM, a concentration 17% of the DB75 found in the wild type line. Concentrations of DB75 in the $DB75^{R}$-CL1 and B48 lines at 8 h were 20% and 23% of wild type concentrations, respectively.

In vitro accumulation after exposure to 500 nM DB75. The wild type S427 line accumulated DB75 to a concentration of 1.0 ± 0.2 mM after 8 h (Figure 3.1B). Concentrations in the *TbAT1-/-* line were approximately 600 μM at 8 h. This concentration represents only 54% of the wild type accumulation. The DB75^{R}-CL1 line accumulated less than 400 μM at 8 h, only 41% of the wild type. Accumulation of DB75 in the B48 line was approximately 66% of wild type accumulation after 8 h. As seen in Figure 3.1B, there was no substantial increase in trypanosome concentrations over time in any of the lines investigated.

In vitro accumulation after exposure to 7.5 μM DB75. At the highest concentration investigated, all four lines accumulated DB75 concentrations close to 2 mM after 1 h, even when trypanosomes lacked P2 transporter activity (Figure 3.1C). At the later time points investigated, a larger difference was seen between the wild type and resistant lines, as concentrations in the S427 increased over the 8 h. Concentrations in the resistant lines did not increase much over the 8 h investigated. Accumulation in the wild type S427 line at 8 h was 6.3 ± 0.8 mM, while concentrations in the resistant lines were around 2-3 mM. *TbAT1-/-* accumulated approximately 55% of the DB75 found in the wild type line. DB75^{R}-CL1 accumulated the least amount of DB75 at 8 h, only 37% of the wild type accumulation. The B48 line accumulated approximately 40% of wild type concentrations.

In vitro Distribution of DB75 in Trypanosomes. A time course of DB75 distribution in all four lines of trypanosomes was determined over 8 h using a concentration of 500 nM. Despite large differences in the fluorescence intensity of DB75 in the four lines (as determined by exposure time), care was taken to ensure that pictures were taken at an exposure that yielded similar intensity in the micrographs in order to make a better comparison between the four lines. The resistant lines needed to be exposed to UV light for

a longer period of time to see fluorescence equivalent to that seen in the wild type line at a much shorter exposure time.

In the wild type S427 line, DB75 rapidly localized to the kinetoplast and nucleus, the two DNA containing organelles in trypanosomes, within 15 min (Figure3.2-S427). By 2 h, DB75 appeared faintly in the acidocalcisomes, which were much brighter by 4 h. By 6 and 8 h, fluorescence in the kinetoplast and acidocalcisomes was bright enough to overwhelm the appearance of nuclear fluorescence.

The distribution to organelles in the resistant lines (Figure 3.2 labeled by trypanosome line name), though similar, took longer to occur than in the wild type line. When 500 nM DB75 was incubated with the *TbAT1-/-* line, the compound was barely visible in the kinetoplast at 15 min (Figure 3.2), but was found only in this organelle until 4 h, when the DB75 was also localized in the nucleus. Acidocalcisomes were faintly visible at 8 h, but only a small number of the organelles appeared to fluoresce yellow and contain DB75. DB75 was localized to the kinetoplast after 15 min of incubation with the DB75^{R}-CL1 line (Figure 3.2), and appeared much brighter in this organelle than in the *TbAT1-/-* line. As with the *TbAT1-/-* line, in the DB75^{R}-CL1 line, DB75 was localized to both the nucleus and kinetoplast by 4 h, and appeared in the acidocalcisomes by 8 h. It qualitatively appeared that DB75 accumulated more in the acidocalcisomes at this time point than in *TbAT1-/-*. With the B48 line, again DB75 appeared first in the kinetoplast by 15 min (Figure 3.2), and spread to the nucleus as well by 2 h. A few acidocalcisomes began fluorescing starting at 6 h. The longer amount of time needed for compounds to accumulate in the nucleus and acidocalcisomes may be related to results showing that one needs to incubate the resistant

lines with DB75 for longer periods of time in order to kill the resistant trypanosomes (Table 2).

In vivo Efficacy of DB75 in Trypanosomes. The in vivo efficacy of DB75 against the wild type S427 and three resistant lines was determined at three doses administered intraperitoneally (IP) – 5 μmol/kg, 15 μmol/kg, and 45 μmol/kg (Table 3.3). The 5 μmol/kg dose was unable to cure any resistant lines. Actually, all three resistant lines were unable to clear parasitemia after this dose was administered. DB75 was able to cure 1 of 3 test mice infected with the S427 wild type line, however. One mouse relapsed 26 days post treatment, and was sacrificed due to a high parasitemia burden on day 32 (>>100 parasites/40X field), while the second mouse relapsed on day 57 post treatment, and died one day later. Mice that appear lethargic and have very high parasitemia, like the mouse described above, are unlikely to self-cure the infection and are euthanized to prevent suffering.

At the 15 μmol/kg dose level, all of the mice infected with S427 trypanosomes have survived, with no relapses for 60 days (Table 3.3). 2 of 3 *TbAT1-/-* infected mice treated with this dose of DB75 survived for 60 days without relapse; the third mouse did not clear the initial parasitemia. Only 1 mouse infected with $DB75^{R}$-CL1 trypanosomes survived for the 60 day test period with no relapses. The other two mice in this test group were unable to clear the initial parasitemia after treatment with 15 μmol/kg DB75. Mice infected with B48 trypanosomes, on the other hand, survived for 60 days with no relapse (Table 3.3).

When treated with a 45 μmol/kg dose of DB75, 2 of 3 mice infected with S427 wild type trypanosomes have survived for greater than 50 days without relapsing. One mouse died almost 40 days into the test period, but died in between parasitemia measurement periods. Therefore it is unable to conclusively state whether this death was due to a relapse

of the infection. However, mice infected with the *TbAT1-/-* or DB75^{R}-CL1 lines, so far only have 2 of 3 surviving mice (Table 3.3). With both of these lines, one mouse was unable to clear the initial parasitemia, but the other mice were able to survive for greater than 47 days. Each of the mice infected with B48 trypanosomes were able to clear parasitemia and have not relapsed after 60 days (Table 3.3).

In vivo Accumulation of DB75 in Trypanosomes and Plasma. DB75 concentrations were determined in trypanosomes isolated from infected mice after a dose of either 5 μmol/kg or 15 μmol/kg IP was administered. Plasma concentrations of DB75 were also determined at each time point.

Accumulation of DB75 in trypanosomes and plasma after 5 μmol/kg dose IP. Concentrations of DB75 in all four trypanosome lines were determined at the low dose of 5 μmol/kg IP (Figure 3.3 - Figure 3.6). As seen with the efficacy experiments above, this dose is partially curative (1/3) for the wild type S427 line, but was unable to cure mice infected with any of the three resistant lines, and was actually insufficient to clear initial parasitemia.

In mice infected with the wild type S427 line, the peak plasma concentration, seen at 15 min after DB75 was administered was 1.6 ± 0.4 μM. The concentration of DB75 in plasma at 24 h was 0.028 ± 0.007 μM. DB75 accumulated to a peak concentration of 800 ± 600 μM in trypanosomes at 4 h, before declining to a concentration of 16 ± 4.6 μM at 24 h (Figure 3.3A). The peak concentration in trypanosomes is similar to peak concentrations seen after 7.5 μmol/kg DB75 is administered IV to infected mice (11). Area Under the Curve over 24 hours ($AUC_{0\text{-}24h}$) for S427 trypanosomes after the 5 μmol/kg dose was over 5000 μM*h, while the plasma AUC was only 3 μM*h (Table 3.4). This represents a 1600 fold greater accumulation in trypanosomes than plasma over the investigated time period.

The peak plasma concentration of DB75 in *TbAT1-/-* infected mice was 2.1 ± 0.4 μM after 15 min, and at 24 hours, plasma concentrations were 0.022 ± 0.003 μM, again similar to concentrations in the wild type line. Concentrations of DB75 in *TbAT1-/-* peaked at 114 ± 94 μM at 8 h after administration (Figure 3.4A). 24 h after DB75 was administered trypanosome concentrations were 16 ± 2.6 μM, similar to 24 h concentrations seen with the wild type S427 line. The AUC of DB75 in *TbAT1-/-* trypanosomes was approximately 830 μM*h, only 16% of the AUC of DB75 in S427 trypanosomes (Table 3.4). Plasma AUC of DB75 in the *TbAT1-/-* line was similar to that seen with the S427 infection (Table 3.4). The ratio of trypanosome AUC to plasma AUC was approximately 270.

In mice infected with $DB75^{R}$-CL1, the peak plasma concentration at 15 min was 2.1 ± 1.1 μM, and the concentration of DB75 in plasma at 24 h was 0.05 ± 0.008 μM. In the $DB75^{R}$-CL1 line, the peak concentration in trypanosomes was 50 ± 25 μM 1 hour after DB75 was administered (Figure 3.5A). By 24 h, trypanosome concentrations had declined to 2 ± 0.04 μM, 8 fold lower than the 24 h trypanosome concentration in the S427 and *TbAT1-/-* lines. The AUC of DB75 in the DB75R-CL1 line was 270 μM*h, only 5% of the AUC seen in S427 trypanosomes. Again, the AUC found in plasma was similar to that of the S427 and *TbAT1-/-* trypanosomes. The AUC ratio for trypanosomes to plasma was about 70 fold for the $DB75^{R}$-CL1 infection.

Mice infected with B48 trypanosomes had plasma concentrations that were higher than in the other three lines at all time points examined. After a peak concentration in plasma of 2.4 ± 0.6 μM at 15 min, a low concentration of 0.13 ± 0.04 μM was found in plasma at 24 h. This plasma concentration is between 2.6 and 6.5 fold higher than the 24 h plasma concentration determined in the other three lines. DB75 also accumulated the least in the

B48 line of trypanosomes. The peak trypanosome concentration was only 17 ± 5 μM, and occurred 12 hours after the compound was administered (Figure 3.6A). Concentrations in trypanosomes did not decline much at 24 h, with a concentration of 13 ± 10 μM seen in trypanosomes at that time. The AUC of DB75 in B48 trypanosomes was approximately 320 μM*h, 6% of the AUC in S427 trypanosomes. The plasma AUC was determined to be 4.9 μM*h, which was higher than plasma AUC values determined for the other three lines investigated (Table 3.4). The AUC ratio for B48 trypanosomes to plasma was about 60 fold.

Accumulation of DB75 in trypanosomes and plasma after 15 μmol/kg dose IP. The 15 μmol/kg IP dose is curative for the wild type S427, and the B48 line. This dose is the approximate ED_{50} dose for *TbAT1-/-* and $DB75^{R}$-CL1.

The peak plasma concentration was 15 ± 9 μM, 15 min after DB75 was administered to mice infected with S427 trypanosomes. The peak trypanosome concentration in the S427 line after the 15 μmol/kg dose was 3289 ± 1236 μM at 2 h (Figure 3.3B). This concentration was almost 4 fold higher than the peak concentration seen after the 5 μmol/kg dose was administered. By 24 h, trypanosome concentrations had only declined to about 400 μM. The AUC of DB75 in trypanosomes was approximately 18000 μM*h, which is a little more than 3 fold higher than the AUC in trypanosomes after the 5 μmol/kg dose. The plasma AUC was 22 μM*h (Table 3.4). There was almost 800 fold AUC trypanosome/plasma ratio with the S427 infection.

The peak plasma concentration in mice infected with the *TbAT1-/-* line was similar to that found in the S427 line, with a plasma concentration of 17 ± 4 μM after 15 min (Figure 3.4B). For the *TbAT1-/-* line, peak trypanosome concentrations were 380 ± 280 μM 1 h after administration, a concentration 3 fold the peak concentration seen with the lower dose. This

peak concentration was also approximately 10 fold lower than the S427 peak concentration. Trypanosome concentrations at 24 h were 26 ± 15 μM. The trypanosome AUC in the *TbAT1-/-* was 3500 μM*h, 20% of the AUC determined for S427 trypanosomes (Table 3.4). Plasma AUC was similar to that of the S427 infected mice at the 15 μmol/kg dose. Trypanosome to plasma AUC ratio for *TbAT1-/-* infected mice was about 160 fold.

In mice infected with DB75^{R}-CL1 trypanosomes, plasma concentrations peaked at 29 ± 9 μM after 15 min, double the concentration seen in the plasma of S427 and *TbAT1-/-* trypanosomes (Figure 3.5B). At 24 h, 2 of the 3 mice investigated had plasma concentrations of DB75 that were below the limit of quantification by HPLC with fluorescence detection, which was surprising given the low trypanosome concentrations at 24 h. A peak trypanosome concentration of 430 ± 350 μM DB75 occurred after 1 hour. This peak concentration is 9 fold higher than the peak trypanosome concentration after the 5 μmol/kg dose. Trypanosome concentrations decline to about 16 ± 15 μM at 24 h (Figure 3.5B). DB75^{R}-CL1 AUC was approximately 3000 μM*h, 17% of the AUC seen in S427 trypanosomes (Table 3.4). Plasma AUC was 32 μM*h, which was slightly higher than seen with either the S427 or *TbAT1-/-* infected mice. The ratio of DB75^{R}-CL1 AUC to plasma AUC was almost 90 fold.

The peak plasma concentration in mice infected with B48 trypanosomes was 31 ± 12 μM, similar to plasma concentrations from DB75^{R}-CL1 infected mice. As with the low dose, DB75 peaked in B48 trypanosomes at 12 h, with at concentration of 146 ± 75 μM. The peak concentration in trypanosomes after the 15 μmol/kg dose was 9 fold higher than after the 5 μmol/kg dose. After the peak, concentrations in trypanosomes declined to 40 ±18 μM at 24 h (Figure 3.6B). The AUC in B48 trypanosomes was determined to be 2000 μM*h, which is

11% of the S427 AUC (Table 3.4). Similar to the DB75^{R}-CL1 infected mice, the plasma AUC for the B48 infected mice was approximately 30 μM*h. The ratio of trypanosome to plasma AUC was approximately 65 fold. As mentioned previously, the concentrations achieved in the B48 trypanosomes were sufficient to clear parasitemia and cure the infection (Table 3.3).

Additionally, if the concentration vs. time profile is normalized for the dose administered, differences were seen. For all of the four lines, overall concentrations in trypanosomes did not increase linearly with increasing dose. For the wild type S47 and *TbAT1-/-* lines, the Cmax concentrations normalized for dose did increase linearly, but it must be cautioned that the Tmax shifted with increasing dose, especially with the *TbAT1-/-* line (8 h to 1 h). In terms of AUC in relation to dose, the trypanosome AUC in the S427 wild type line and the *TbAT1-/-* line increased approximately linearly with increasing dose, although both lines had a slightly higher AUC at the 15 μmol/kg dose than would be predicted based on the 5 μmol/kg dose. For the DB75^{R}-CL1 and B48 lines, the trypanosome AUC increase with increased dose was nonlinear, as the AUC values determined at the higher dose were 7-11 times higher than the AUC found at the lower dose (Table 3.4). Plasma concentrations also increased nonlinearly with increasing dose, as the peak concentrations and AUC were much higher with the 15 μmol/kg dose in comparison to the 5 μmol/kg dose for all lines investigated.

In vivo Distribution of DB75 in Trypanosomes. In vivo distribution of DB75 in all four trypanosome lines was similar to that seen in vitro. Distribution of DB75 in each trypanosome line after both 5 μmol/kg and 15 μmol/kg doses is presented.

Intracellular distribution after 5 µmol/kg dose. After a 5 µmol/kg dose administered to mice infected with the wild type S427 strain, DB75 appeared in the nucleus and kinetoplast after 15 min. By 1 – 2 h, acidocalcisomes were becoming fluorescent as well. Fluorescence in the acidocalcisomes became more intense over the 24 h period investigated. By 24 h, DB75 appeared to primarily be localized only in the nucleus and acidocalcisomes (Figure 3.7).

In the three resistant lines, DB75 appeared in the kinetoplast after 15 min (Figure 3.7). DB75 then localized to the nucleus, as well as the kinetoplast, over time in each of the lines. By 1 h, DB75 appeared in the nucleus of *TbAT1-/-* trypanosomes, but faded over time. By 24 h, DB75 was localized to only the kinetoplast in *TbAT1-/-* trypanosomes. For the $DB75^{R}$-CL1 line, DB75 was localized to the nucleus by 2-4 h, and like *TbAT1-/-* trypanosomes, appeared only in the kinetoplast by 24 h. In the B48 line, DB75 appeared in the nucleus by 4 h, but by 8 h was again found only in the kinetoplast (Figure 3.7).

Intracellular distribution after 15 µmol/kg dose. In the wild type S427 line, DB75 was localized to the kinetoplast and nucleus 15 min after administration of the compound IP (Figure 3.8). By 2 hours, DB75 was localized to the nucleus, kinetoplast and acidocalcisomes, a pattern which remained the same over the 24 h investigated.

In the resistant lines, DB75 appeared in the kinetoplast by 15 min, and sometimes very faintly in the nucleus at this time as well (Figure 3.8). Between 2 and 4 h, DB75 spread to the nucleus as well as the kinetoplast for all three resistant lines. Distribution in the acidocalcisomes was found at varying times in the resistant lines – between 4 and 8 h. At later time points, the three lines differed in their distribution profiles, perhaps related to differing concentrations in these lines at the later time points. DB75 is found in the nucleus, kinetoplast and acidocalcisomes at 24 h in the *TbAT1-/-* line, but was only faintly localized in

some of these organelles for the DB75^{R}-CL1 and B48 lines. DB75 appears very faint in these two lines at 24 h (Figure 3.8).

E. Discussion

Drug resistance is an important consideration for any new potential therapy, but is especially important for new drugs for trypanosomiasis. Only a few treatments are currently available for treating trypanosomiasis, with melarsoprol already associated with drug resistance and treatment failures. Diamidine compounds like pentamidine and DB75 are accumulated in trypanosomes through uptake transporters on the surface of the cell (4, 5, 10). Uptake of a diamidine through more than one transporter has been shown to be beneficial in the past. Pentamidine is accumulated through three transporters and has shown little clinical resistance despite extensive use (5). Melarsoprol and diminazene, on the other hand, are thought to be transported into trypanosomes primarily by one transporter, and have been associated with drug resistance (6, 12, 13). Additionally, several clinical isolated strains that are drug resistant have been found to have mutated drug uptake transporters (13).

Several trypanosome lines have been created which lack uptake transporters or transporter activity. *TbAT1-/-* and the DB75^{R}-CL1 are both missing DNA containing the gene for the P2 transporter (10, 14). The B48 line, created as a pentamidine resistant line from *TbAT1-/-*, lacks both the P2 transporter and HAPT1 activity (3). Past studies have suggested that the P2 transporter is the primary means for accumulation of DB75 in trypanosomes, and that without this transporter, short term uptake of DB75 was almost nonexistent (10). However, investigations here have shown that accumulation of DB75 still occurs in trypanosomes lacking the P2 transporter over longer periods of time both in vitro and in vivo.

In vitro activity of DB75 against wild type S427, *TbAT1-/-*, and DB75^{R}-CL1 lines determined here were lower than previously reported (10), but the fold resistance is similar to

the previous work. B48, which also lacks HAPT1 activity, has a similar resistance profile to the other two resistant lines, indicating that HAPT1 does not play a major role in the uptake of DB75 into trypanosomes. However, there were large differences in the time that DB75 needs to be exposed to the different trypanosome lines in order for killing to occur. With the wild type line, DB75 only needs to accumulate in trypanosomes for an hour (from accumulation data, this would be an accumulation of 1-2 mM). In the resistant lines, DB75 needs to accumulate in the cells for much longer periods of time in order to cause trypanosome death. This could be due to differences in intracellular distribution, and that the resistant lines appear to need much longer times to achieve similar intracellular distribution patterns to the wild type line.

Surprisingly, accumulation of DB75 in the lines lacking uptake transporters was high (μM to mM concentrations), as seen in Figure 3.1. At the lowest concentration of DB75 investigated, 150 nM, we saw the largest difference in accumulation between the wild type and resistant lines. In the S427 line, DB75 concentrations increased over the 8 h period investigated, while concentrations in the other lines investigated reached a plateau between 50 and 100 μM, about 20% of wild type S427 concentrations. At the higher doses, the gap between the wild type and resistant line concentrations became smaller, with the resistant lines accumulating 40-60% of the DB75 found in the wild type line. The higher concentrations may have been overwhelming to trypanosome transporters, which could have reduced the effect of the line differences. It is possible that at higher concentrations of DB75 other uptake processes may have taken over in the resistant lines, allowing for increased accumulation at the higher dose levels. Perhaps there is an unknown low affinity, high capacity transporter capable of accumulating DB75.

The discrepancies in the in vitro accumulation experiments and previous experiments detailing short term uptake rates bear further investigation. Extrapolating from the rate determined by Lanteri et al. for uptake in *TbAT1-/-* and DB75^{R}-CL1 lines (approximately 0.01 pmol/10^{7}cells/min (10)), concentrations of DB75 in trypanosomes after 8 h would be 8.3 μM after a 1 μM dose, yet after 500 nM DB75, a concentration of almost 400 μM was seen. Therefore, it is unlikely that one can extrapolate from short term experiments to longer ones. It would be prudent to investigate uptake and accumulation over short and long periods of time.

The in vitro experiments performed in this study were performed in CBMEM media, while short term accumulation experiments were conducted in Carter's Balanced Salt Solution (CBSS) (10). Additionally, in the short term experiments, trypanosomes are usually isolated from rats, and used ex vivo. There may be some differences between trypanosomes in vitro and in vivo. Although the media contains more protein, it is unclear if there is any advantage to DB75 accumulation in trypanosomes in the presence of serum proteins. Further experiments are planned in which accumulation is investigated in several serum free media and buffer systems.

Additionally, CBMEM media contains high concentrations of adenosine and other purines. The concentration of adenosine in the media is approximately 80 μM. Adenosine has been shown to inhibit DB75 uptake into trypanosomes with an IC_{50} value of 0.8 μM (9). With concentrations in media 100 fold above the concentration necessary to inhibit DB75 uptake by 50%, it is possible that the P2 transporter is saturated. Therefore, accumulation of DB75 that occurs in this media system may be largely independent of the P2 transporter. It is unknown whether adenosine's ability to inhibit DB75 accumulation extends over time or if it

is a short term phenomenon. Furthermore, DB75 has a similar affinity for the P2 transporter and has been shown to inhibit adenosine uptake into trypanosomes with a similar IC_{50} value (9). However, this does not explain why accumulation of DB75 is still reduced in the absence of the P2 transporter. This puzzling finding needs further investigation. One research strategy may be to implement a stepwise change in the media/buffer system – from the CBMEM media to a buffer system like CBSS, with accumulation of DB75 measured with each change. The first step would be to remove adenosine alone from the media, leaving other purines (for example, hypoxanthine) in the media which are not substrates for the P2 transporter. A gradual decrease in the amount of fetal bovine serum (FBS) could be the next step, with the final media containing no protein. Then a change to various buffer systems, with and without serum proteins can be used to determine accumulation of DB75.

In vitro distribution studies showed DB75 accumulation (at a 500 nM dose) in the kinetoplast of *TbAT1-/-*, DB75R-CL1, and B48 after 15 min. This concentration is much lower than ones previously used before (10, 17). Although DB75 is found in the kinetoplast after 15 min in all three resistant lines, the distribution pattern spreads over a much longer time course than in the wild type line. It is not until 8 h that the resistant lines show the same distribution that is found in the wild type line after 1-2 h. This delayed distribution could explain the need for longer incubation times with DB75 to induce killing in the resistant lines in vitro.

In vivo efficacy of DB75 against the S427 line was similar to previous reports (10), where a 4 X 0.5 mg/kg dose cured infected mice. In the *TbAT1-/-* and $DB75^{R}$-CL1, DB75 was able to cure infections after a dose of 4 X 2.5mg/kg and 4 X 1 mg/kg respectively. The cumulative dose administered to wild type trypanosomes in the previous report was 2 mg/kg,

equivalent to the 5 μmol/kg dose administered in these experiments, which cured 2 of 3 mice infected with S427 trypanosomes in this investigation. The 15 μmol/kg dose cured all animals infected with S427 trypanosomes and B48 trypanosomes, but only partially cured *TbAT1-/-* and $DB75^{R}$-CL1 lines. Similar response was seen after a 45 μmol/kg dose was administered. Efficacy of DB75 in all strains needs to be examined in larger test groups in order to have sufficient power to determine differences between dose levels administered.

One discrepancy between our results and previous data is in relation to the in vivo virulence and infectivity of B48. Bridges et al. report that in their hands, B48 was unable to produce a virulent infection in ICR mice, with self – cures occurring even when mice were immunosuppressed with cyclophosphamide before infection (3). Berger et al. also developed a pentamidine resistant line under laboratory conditions and found that it had reduced virulence in Tyler's original mice. However, by increasing the concentration of trypanosomes injected into mice 250 fold, they were able to see equivalent survival time (2). It has been suggested that the acquisition of pentamidine resistance has led to decreased virulence of both of these lines, but in our hands, we saw no decreased virulence when Swiss Webster mice were infected with the B48 line. Our experiments did use a higher inoculum (10^5 trypanosomes compared to 10^4 trypanosomes) than that of Bridges et al. (3). At this infection concentration, we saw similar virulence in terms of parasitemia and mouse death due to the infection. It is unknown at this time whether the ICR mice and Swiss Webster mice react differently to this parasite, or if there is some other cause for the differences in virulence of the B48 line.

In vivo accumulation of DB75 in the trypanosome lines investigated yielded interesting results. At the lowest dose investigated, 5 μmol/kg DB75, peak concentrations in

the S427 wild type trypanosomes were very similar to concentrations seen previously with a 7.5 μmol/kg IV dose (11). However, the resistant lines concentrations were greatly reduced. Peak accumulation of DB75 in *TbAT1-/-* trypanosomes was 14% of S427 accumulation, and was only 6% and 2% in $DB75^R$-CL1 and B48, respectively, after the 5 μmol/kg dose. These low concentrations are likely to be why this dose was insufficient to cure mice infected with the resistant lines. This indicates that we may need to be concerned with under dosing of DB75 leading to a greater chance of drug resistance in patients.

What was really interesting though was the lack of dose proportionality in trypanosome and plasma concentrations with the two doses. While the peak concentration of DB75 in S427 trypanosomes at the 15 μmol/kg dose was a little more than 3 fold higher than after the 5μmol/kg dose, in two of the resistant lines, peak concentrations were 9 fold higher at the higher dose ($DB75^R$-CL1 and B48). Like the S427 line, *TbAT1-/-* concentrations were 3 fold higher at the 15 μmol/kg dose compared to the 5 μmol/kg dose. Peak plasma concentrations after the 15 μmol/kg dose were also much higher than that seen after the 5 μmol/kg dose was administered. Plasma AUC at the higher dose was 7-8 fold higher than seen at the lower dose for mice infected with any of the 4 lines, despite only a 3 fold increase in dose. Additionally much lower concentrations were able to cure the B48 trypanosome infection compared to the other resistant lines.

The studies described here are the first to investigate long term accumulation of diamidines like DB75 in trypanosomes which lack the P2 transporter, and are therefore resistant to DB75. Short term accumulation experiments have indicated minimum accumulation in the DB75R-CL1 and *TbAT1-/-*, but nonetheless, the authors of those experiments found that DB75 was still able to kill these trypanosomes in vitro, albeit over a

longer period of time (10). Lanteri et al. were unable to grow the DB75R-CL1 line in vitro in the presence of DB75 for longer than 4 days, even at concentrations well below that with which resistance was selected (10). The studies here have shown that this is due to accumulation in these resistant lines, perhaps by some unknown uptake transporter. Although the distribution in all three of these resistant lines spreads slower throughout the trypanosome, by 8 h in vitro similar distributions are seen in drug resistant and wild type trypanosomes. In vivo accumulation of DB75 in resistant trypanosomes was much lower, but in some lines, concentrations were high enough to cure infection. In vivo distribution in the resistant lines never achieved the distribution seen in the wild type line. This is likely related to the much lower concentration of DB75 found in the three resistant lines.

Much remains to be studied with drug resistance and DB75, especially since DB289 will likely be available for clinical use in a few years. Whether clinical resistance to this compound will be a factor in the future still needs to be determined. Melarsoprol resistant lines isolated from patients in the clinic have been shown to have defective P2 transporters (with mutated amino acids) (13). To our knowledge accumulation and distribution of DB75 over long periods of time has not been investigated in any of these clinical lines. Since the resistant lines created in the laboratory actually have the entire genetic sequence of the P2 transporter removed, it is unknown how well these lines will actually predict the many forms of mutations that may be seen in patients due to drug resistance. Additionally, it seems imperative that the accumulation and distribution of DB75 in these drug resistant trypanosomes be determined after the administration of the prodrug DB289. DB75 concentrations in plasma after administration of DB289 are not as high as that seen after administration of the active drug alone, and this may lead to lower concentrations in drug

resistant trypanosomes. Determining concentrations in trypanosomes during a multiple dose administration regimen will also allow us to investigate the impact of drug resistance in these resistant lines in a more clinically relevant manner, as DB289 will be administered as on a 5 day dosing schedule.

F. Acknowledgements

The authors would like to thank Drs. Mike Barrett and Harry de Koning for the generous donation of *TbAT1-/-,* DB75^{R}-CL1, and B48 lines.

Table 3.1: In vitro Antitrypanosomal Activity of DB75 Against Wild Type Line and Resistant Lines Lacking Uptake Transporters

Line	IC_{50} value (nM)	Fold Resistance
S427 (wild type)	6.5 ± 5.7	
TbAT1-/-	179.6 ± 34.7	25
$DB75^{R}$-CL1	200 ± 71.3	28
B48	159.6 ± 30.6	31

Table 3.2: Time of Incubation Necessary for DB75 to Kill Trypanosomes in vitro				
	S427	TbAT1-/-	$DB75^{R}$-CL1	B48
500 nM	1 h	24 h	>48 h	48 h
7.5 μM	1 h	4-8 h	8-24 h	8-24 h

Table 3.3: In vivo Efficacy of DB75 Against Wild Type Line and Lines Lacking Uptake Transporters at Three Doses

Line	Dose	Cure Rate	MST (days)
S427	45 µmol/kg IP	2/3	>55
	15 µmol/kg IP	3/3	60
	5 µmol/kg IP	1/3	49
TbAT1-/-	45 µmol/kg IP	2/3*	>33
	15 µmol/kg IP	2/3*	41
	5 µmol/kg IP	0/3	4
$DB75^{R}$-CL1	45 µmol/kg IP	2/3*	>33
	15 µmol/kg IP	1/3*	23
	5 µmol/kg IP	0/3	5
B48	45 µmol/kg IP	3/3	>53
	15 µmol/kg IP	3/3	60
	5 µmol/kg IP	0/3	6

MST = mean survival time in days, cure rate represents animals surviving without parasitemia

*Animal(s) died before clearing initial parasitemia, and the survivors did not relapse over the 60 day test period.

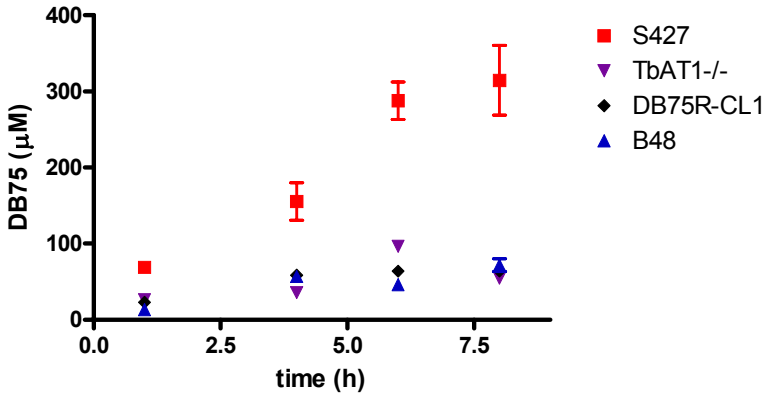
Table 3.4: Pharmacokinetic Analysis of DB75 in Trypanosomes and Plasma

		S427		TbAT1-/-		DB75^{R}-CL1		B48	
Dose		AUC μM*h	Ratio[1]	AUC μM*h	Ratio	AUC μM*h	Ratio	AUC μM*h	Ratio
5 μmol/kg	Plasma	3.3		3.0		3.7		4.9	
	trypanosomes	5200	1600	830	270	270	70	320	60
15 μmol/kg	Plasma	22		22		32		32	
	trypanosomes	18000	800	3500	160	3000	90	2100	65

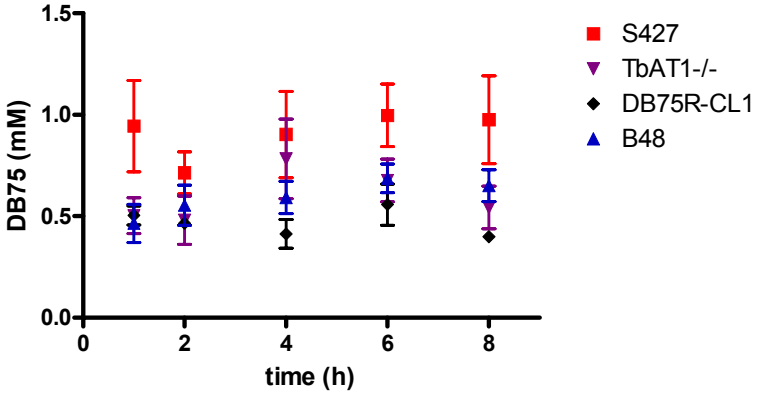
[1]Ratio of Trypanosome AUC to Plasma AUC

Figure 3.1: In vitro accumulation of DB75 in wild type and drug resistant trypanosomes over time at three concentrations.

A. 150 nM DB75



B. 500 nM DB75



C. 7.5 μM DB75

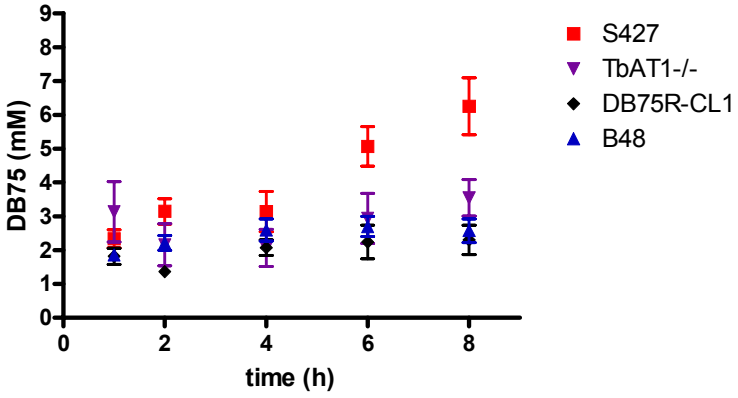


Figure 3.2: in vitro distribution of 500 nM DB75 in the wild type S427 and three resistant lines (*TbAT1-/-*, DB75^{R}-CL1, and B48) over time.

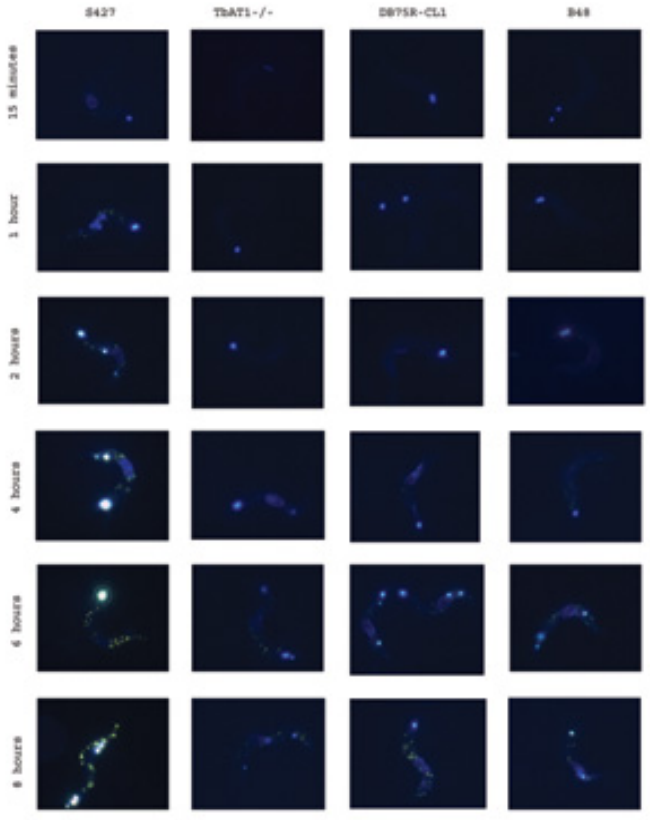
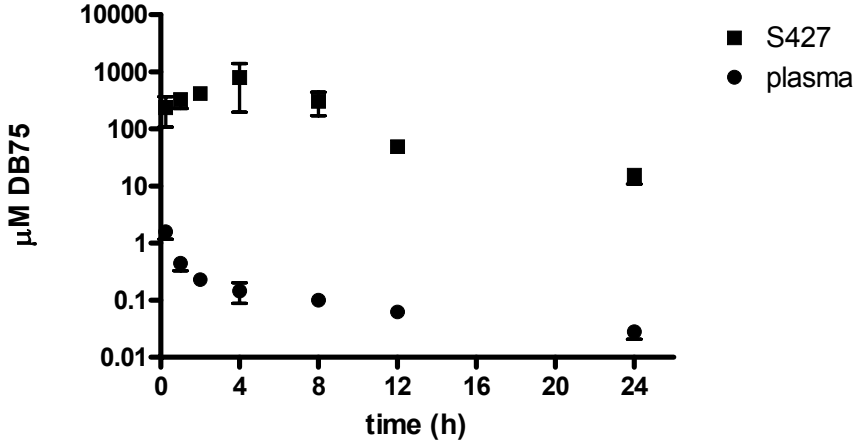


Figure 3.3: Accumulation of DB75 in wild type S427 trypanosomes at a dose of 5 μmol/kg and 15 μmol/kg administered IP.

A. 5 μmol/kg DB75



B. 15 μmol/kg DB75

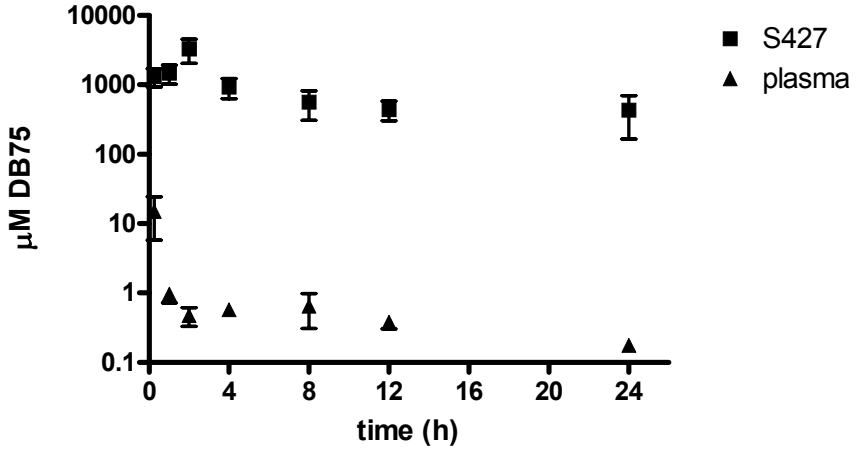
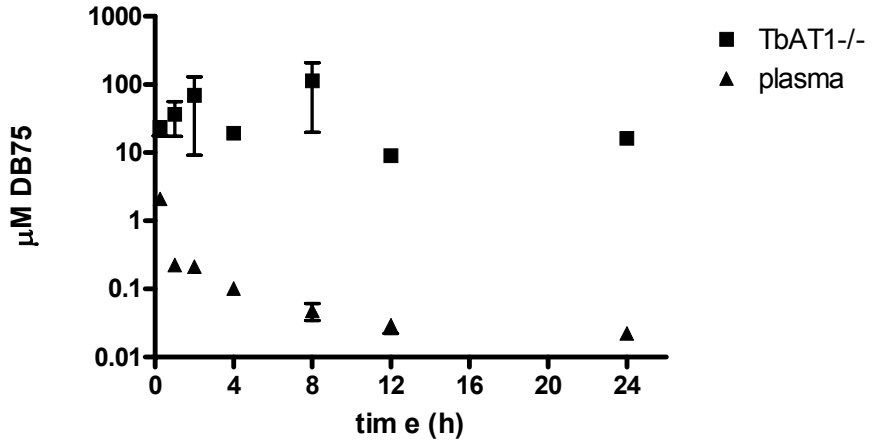


Figure 3.4: Accumulation of DB75 in *TbAT1-/-* trypanosomes after a 5 μmol/kg dose and a 15 μmol/kg dose administered IP.

A. 5 μmol/kg DB75



B. 15 μmol/kg DB75

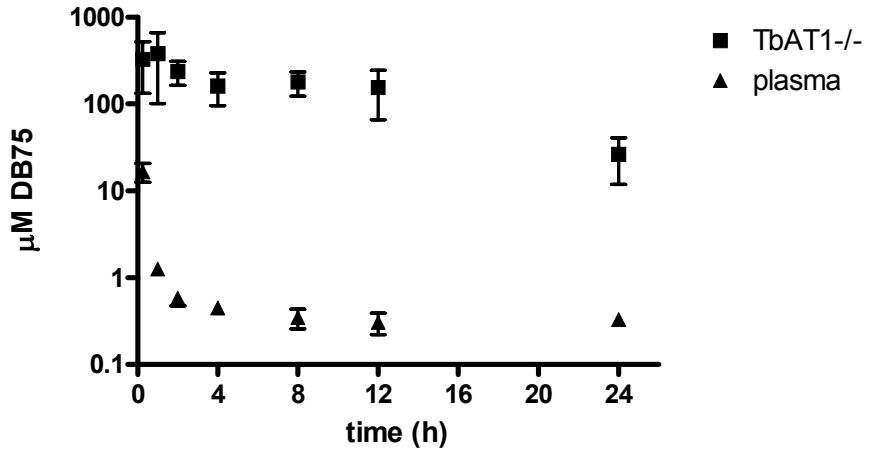
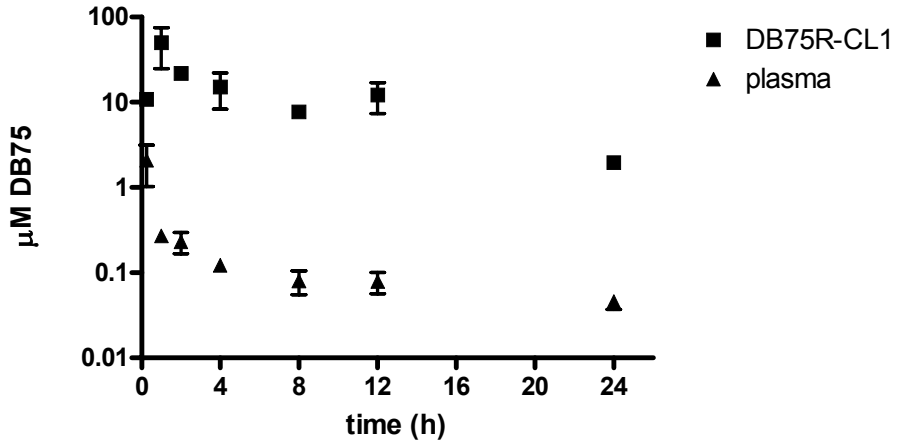


Figure 3.5: Accumulation of DB75 in DB75^{R}-CL1 trypanosomes after a 5 μmol/kg and a 15 μmol/kg dose administered IP.

A. 5 μmol/kg DB75



B. 15 μmol/kg DB75

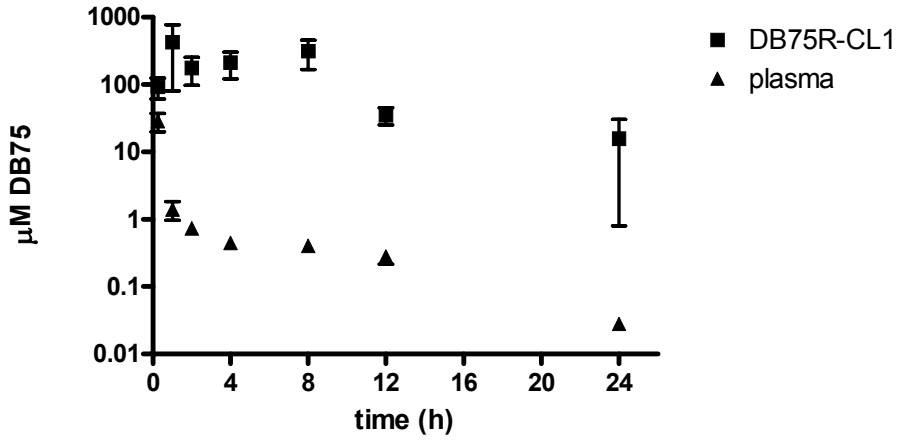
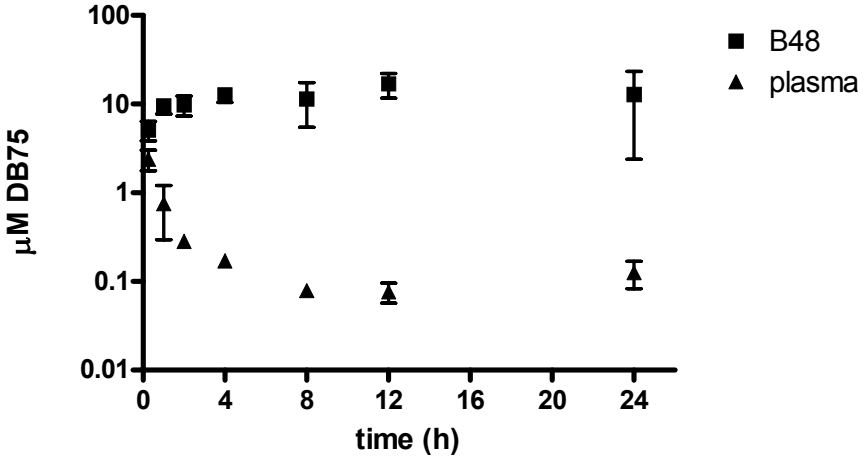


Figure 3.6: Accumulation of DB75 in B48 trypanosomes after a 5 μmol/kg and a 15 μmol/kg dose administered IP.

A. 5 μmol/kg DB75



B. 15 μmol/kg DB75

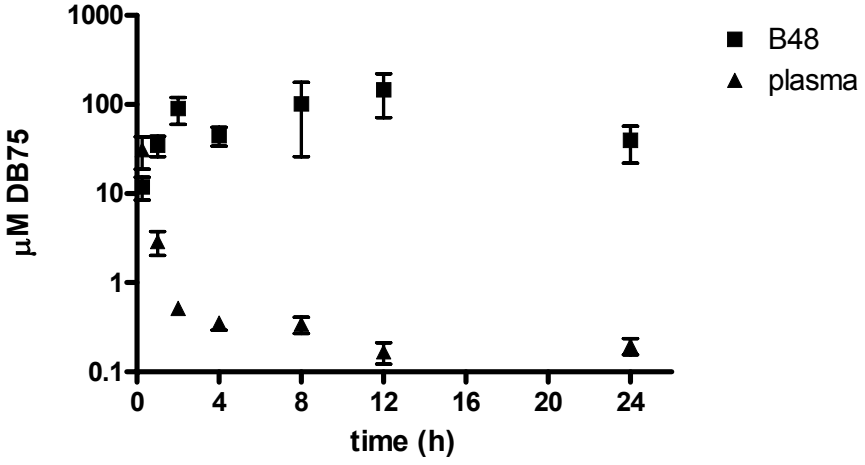


Figure 3.7: in vivo distribution of DB75 in wild type and drug resistant trypanosomes after a 5 μmol/kg dose of DB75 administered IP. Distribution in S427, the wild type line is shown in the left column, with the distribution in the resistant lines in the three right columns (*TbAT1-/-,* $DB75^{R}$-CL1, and B48, respectively).

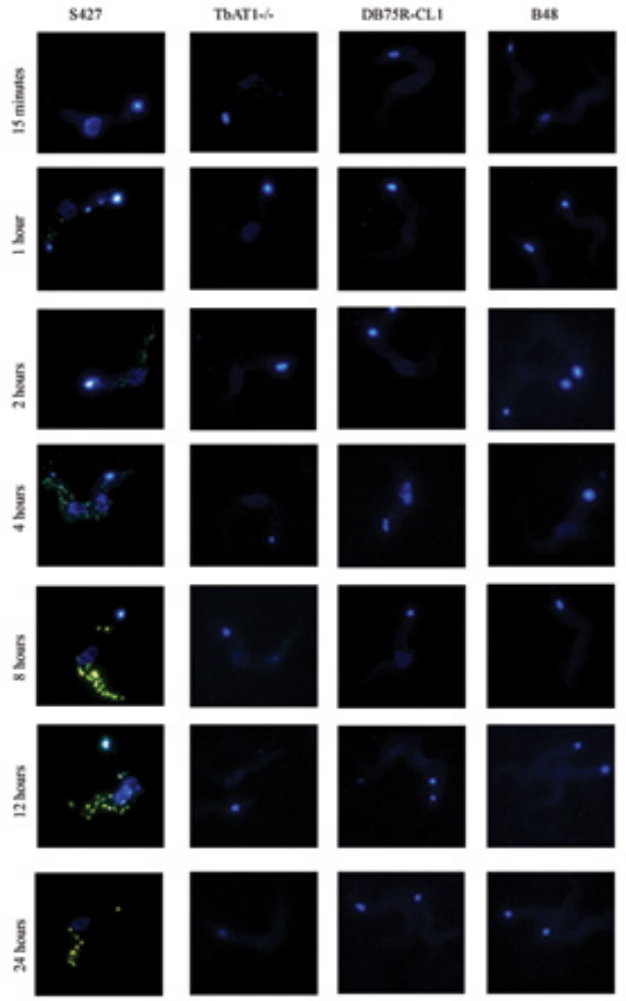
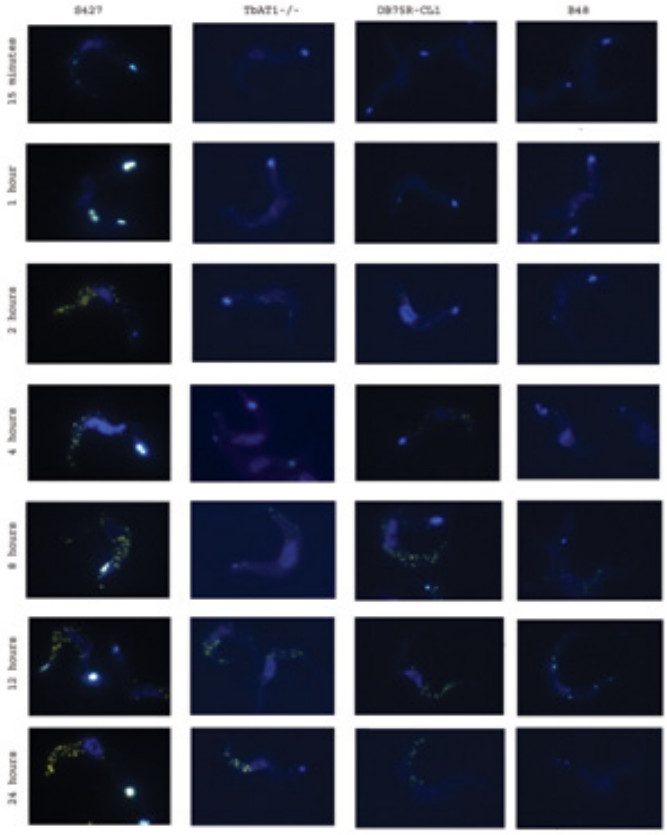


Figure 3.8: in vivo distribution of DB75 in wild type and drug resistant trypanosomes after a 15 µmol/kg dose of DB75 administered IP. Distribution in S427, the wild type line is shown in the left column, with the distribution in the resistant lines in the three right columns (*TbAT1-/-,* DB75^{R}-CL1, and B48, respectively).



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CHAPTER 4

DIPHENYL FURANS AND AZA ANALOGS: EFFECTS OF STRUCTURAL MODIFICATION ON IN VITRO ACTIVITY, DNA BINDING, AND ACCUMULATION AND DISTRIBUTION IN TRYPANOSOMES

This chapter has been accepted for publication in *Antimicrobial Agents and Chemotherapy*.

A. Abstract

Human African trypanosomiasis is a devastating disease with only a few treatment options, including pentamidine. Diamidine compounds such as pentamidine, DB75, and DB820 are potent antitrypanosomal compounds. Previous investigations have shown that diamidines accumulate to high concentrations in trypanosomes. However, the mechanism of action of this class of compounds remains unknown. A long-hypothesized mechanism of action has been binding to DNA and interference with DNA-associated enzymes. The fluorescent diamidines, DB75 and DB820, have been shown to localize not only in the DNA containing nucleus and kinetoplast of trypanosomes, but also to the acidocalcisomes. Here we investigate two series of analogs of DB75 and DB820 with varying in vitro antitrypanosomal activity to determine whether any correlation exists between trypanosome accumulation, distribution and in vitro activity. Despite wide ranges of in vitro antitrypanosomal activity, all of the compounds investigated accumulated to millimolar concentrations in trypanosomes over 8 hours. Interestingly, some of the less potent compounds accumulated to concentrations much higher than more potent compounds. All of the compounds were localized to one or both of the DNA containing nucleus or kinetoplast, and many were also found in the acidocalcisomes. Accumulation in the nucleus and kinetoplast should be important to the mechanism of action of these compounds. The acidocalcisomes also may play a role in the mechanism of action of these compounds. This investigation suggests that the extent of accumulation alone is not responsible for killing trypanosomes and that organelle specific accumulation may not predict in vitro activity.

B. Introduction

Diamidine compounds, such as pentamidine, propamidine and diminazene have been used for many years as chemotherapeutic agents for infections caused by a variety of microbes, including parasites and fungi. Pentamidine has been used for almost 60 years as a treatment for human African trypanosomiasis, and is also used to treat leishmaniasis and the opportunistic infection *Pneumocystis* pneumonia (32). Diminazene has been used widely for animal trypanosomiasis (9), and has also been used for humans (25). Recently pafuramidine, or DB289, a methamidoxime prodrug of the diamidine DB75 (furamidine), has been developed as an oral treatment for early stage sleeping sickness caused by *Trypanosoma brucei gambiense.* DB289 is currently in Phase III clinical trials in sub-Saharan Africa (6). In addition to DB75 and DB289, a library of diamidines and prodrugs has been synthesized, with variable activity against many parasites (1-3, 14-18, 32).

Although diamidines have been used therapeutically for over half a century, their mechanism of action is not well understood. Many mechanisms of action have been proposed (32), but one mechanism of action of diamidines that has often been hypothesized is binding to DNA in the nucleus or kinetoplast, leading to interference of DNA associated enzymes such as Topoisomerase II (27, 34). Pentamidine has also been shown to linearize kinetoplast DNA (kDNA) isolated from trypanosomes (27), which could play an important role in the compound's mechanism of action. Furthermore, ultrastructural studies of whole trypanosomes have demonstrated that diamidines such as pentamidine have an impact on the kinetoplast of trypanosomes, perhaps leading to the disintegration of kDNA (33). However, neither quantitative nor qualitative investigations have been performed to determine in which compartments pentamidine localizes in trypanosomes. This is not surprising, as pentamidine

is not a fluorescent compound, and therefore its intracellular distribution cannot be tracked using microscopy.

Several innately fluorescent compounds related to pentamidine have been investigated qualitatively in cancer cells (19, 20) to determine effects of structural modifications on nuclear distribution. Compounds tested included DB75, and the substituted diamidines DB569, DB244 and DB249, and the imidazoline DB60 (Figure 4.1). Lansiaux et al. found that of the three substituted diamidines, only the N-phenyl substituted DB569 had an altered distribution in cells. While the other compounds were localized to the nuclei of cancer cells, DB569 was found only in the mitochondria (20).

More recently, the accumulation and distribution of two fluorescent analogs of pentamidine, DB75, and its aza analog, DB820, in trypanosomes were described (24). Both compounds accumulated within trypanosomes to millimolar concentrations both in vitro, and in an in vivo mouse model, with concentrations in trypanosomes approximately 1000 fold, or greater, the environmental concentrations. These compounds are distributed not only in the nucleus and kinetoplast, the DNA containing organelles of trypanosomes, but were also found to be localized to acidocalcisomes. Although DNA binding has been hypothesized to be the mechanism of action for diamidines, accumulation in the acidocalcisomes or other organelles may also play an important role in killing these parasites.

This paper describes the in vitro activity, DNA binding ability, accumulation, and distribution for thirteen structurally related compounds. While some are intensely fluorescent and others are not as fluorescent, we have combined qualitative microscopy studies to determine intracellular localization of the compounds with quantitative High Pressure Liquid Chromatography (HPLC) and Liquid Chromatography/Mass Spectrometry

(LC/MS) to determine the concentration of compounds within trypanosomes. The compounds investigated, which have the same core structure, do have several structural modifications that could influence activity and DNA binding, as well as accumulation and distribution within trypanosomes. Here we investigate two series of compounds – diphenyl furans (including DB75) and aza analogs of diphenyl furans (including DB820) to determine whether any correlation exists between trypanosome accumulation and intracellular distribution and in vitro activity.

C. Materials and methods

Antitrypanosomal Diamidines. Diamidine compounds were synthesized as previously described (3, 7, 16, 20). Stock solutions of the compounds were prepared at a concentration of 1 mM in sterile distilled water.

In vitro Antitrypanosomal Activity. To determine IC_{50} values, *T. b. brucei* S427 and *T. b. rhodesiense* STIB900 trypanosomes (2×10^4 per ml) were cultured at 37 ºC and 5% CO_2 with serial dilutions of the diamidines for 72 h using a method previously described (26). The *T. b. brucei* S427 strain is routinely used at UNC for in vitro activity, compound accumulation and intracellular distribution studies. The *T. b. rhodesiense* STIB900 strain is routinely used at the Swiss Tropical Institute for screening diamidine compounds for both in vitro and in vivo activity (15). Experiments were performed in triplicate with each trypanosome strain. IC_{50} values were determined using the Hill Equation, and are the average of at least 3 determinations.

DNA binding by thermal melting. Thermal melting experiments were conducted with a Cary 300 Bio UV-Visible spectrophotometer (Walnut Creek, CA) as previously described (35). DNA samples for melting studies were added to 1 ml of MES buffer (0.01 M MES, 0.1 M NaCl, 0.001 M EDTA, pH 6.2) in 1 cm path length reduced volume quartz cells. Experiments were generally conducted at a concentration of 5×10^{-5} M in base pairs for polydA·polydT or 1×10^{-6} M in duplex for the self-complementary oligomer, $[d(CGCGAATTCGCG)_2]$ (The Midland Certified Reagent Company, Inc., Midland, Texas) which has a single AT binding site for the diamidines. The concentration of DNA was determined by measuring the absorbance at 260 nm. For the diamidine-DNA complexes, a ratio of 1:1 compound to DNA oligomer duplex or a ratio of 0.6 compound/DNA base pairs

with polydA·polydT was used. A thermistor fixed into a reference cuvette was used to monitor the temperature and the heating rate was 0.5 °C/min. The data were collected as absorbance (A) versus temperature (T) by a computer which also controlled the instrument. The melting temperature (T_m) values for DNA alone or for the complex of DNA and compound were determined by first derivative analysis (plot $\Delta A/\Delta T$ versus T) where the maximum marks Tm at the inflection point in the sigmoidal melting curve (A versus T).

$$\Delta T_m = T_m \text{ for complex} - T_m \text{ for DNA}$$

Fluorescence Spectra of Diamidines in Aqueous Solution. Fluorescence scans to determine excitation and emission maxima were performed using a Perkin Elmer Luminescence Spectrophotometer (LS 50B) using FL Winlab Version 4.00.02 software (Perkin Elmer, Wellesley, MA). Compounds were prepared as 500 nM solutions in distilled water. One ml of each solution was transferred to a disposable cuvette (Fisher Scientific) and the fluorescence spectrum was determined. Fluorescence intensity measurements were then determined using the λ_{ex} and λ_{em} maxima.

For some compounds, the fluorescence properties were determined in the presence of DNA. Briefly, fluorescence emission spectra were obtained with a Cary Eclipse spectrofluorometer with software provided to control the instrument and collect the fluorescence data. Typically, the fluorescence intensity for a compound at a concentration of 1 μM was measured at 25 °C in MES buffer. Compounds were excited at their maximum excitation wavelength as determined above and fluorescence emission spectra were collected. A solution of the compound was titrated with aliquots of a stock solution of *Clostridium*

perfringens genomic DNA (Sigma) that contains 72% AT base pairs and the samples were rescanned for the emission spectra.

In vitro Accumulation of Diamidines in S427 Trypanosomes. To determine in vitro accumulation of diamidines within trypanosomes over time, approximately 10^6 trypanosomes were incubated with 7.5 μM of each diamidine for time periods ranging from 1 to 8 h. At each time point, trypanosomes were washed twice in fresh drug-free media, lysed, and extracted in 4 volumes of 8:1 methanol: 0.1 N HCl (vol/vol). Half of each sample was evaporated in a Zymark (Hopkinton, MA) Turbovap-LV under nitrogen gas (7 psi) and reconstituted in High Performance Liquid Chromatography (HPLC) solvent A (described in detail in HPLC Analysis of Diamidines). The remainder of the sample was retained for future analysis if needed. Diamidine concentrations in trypanosomes were determined as described previously (24).

In vitro Distribution of Diamidines in S427 Trypanosomes. S427 trypanosomes were cultured in 24 well plates (Corning Costar, Fisher Scientific) at a starting concentration of 10^5 trypanosomes/ml. Diamidines were added to each well to a final concentration of 500 nM. Trypanosomes were incubated with the diamidines for 8 h at 37ºC, and then trypanosomes were examined under a light microscope for motility and viability. Trypanosomes were washed twice in 1 ml fresh medium which contained no diamidine and resuspended in 20 μl of freshly isolated mouse blood. A small drop of blood was placed on a microscope slide and thin films were prepared. Thin films were used to reduce the photo bleaching that occurs when viewing live trypanosomes under the UV-fluorescence cube. No differences in intracellular distribution of fluorescence were seen when live trypanosomes were viewed under fluorescent light.

Fluorescence Microscopy of Diamidines in Trypanosomes. Thin films were mounted with a drop of glycerol before observation. A Nikon Microphot FXA microscope (Garden City, NJ) with a 60 DM x 1.4 NA objective lens, a mercury lamp, and an Optronics DEI 750 CCD camera (Goleta, CA), was used for fluorescence microscopy. The microscope was equipped with a Nikon UV2A cube that limits excitation wavelengths to 330-380 nm and emission wavelengths to ≥ 420 nm. The UV2A cube allows for the visualization of the blue fluorescence seen in the nucleus and kinetoplast, and the yellow/orange fluorescence seen in the acidocalcisomes due to interactions of diamidines with polyphosphates in these organelles (31).

Distribution of DB75 in the Acidocalcisomes. *Trypanosoma brucei brucei* S427 trypanosomes were cultured in CBMEM media as previously described (24). For various time points from 5 min - 1 h, 10^6 trypanosomes were pretreated with a stock solution of 5 μM monensin (Sigma) prepared in ethanol or 20 mM ammonium chloride. After pretreatment, trypanosomes were washed and then DB75 was added to a final concentration of 7.5 μM. DB75 was incubated with trypanosomes for 2 h, and then trypanosomes were washed twice and resuspended in freshly isolated mouse blood (20 μl). Thin films were prepared from a drop of blood, and viewed with a Nikon Microphot FXA microscope equipped with a 60 DM x 1.4 NA objective lens, a mercury lamp, and QImaging Micropublisher 3.3 CCD Digital Camera (QImaging Corporation, Surrey, BC, CA) was used for fluorescence microscopy. QCapture version 3.0 imaging software was used to capture images (QImaging Corporation). The microscope was equipped with a Nikon UV2A cube that limits excitation wavelengths to 330-380 nm and emission wavelengths to ≥ 420 nm. Acridine orange (Sigma) was also used

as a marker for acidocalcisome accumulation. Acridine orange has been shown in the past to accumulate in acidocalcisomes and be displaced by alkalinizing agents (12).

HPLC Analysis of Diamidines. HPLC analytical methods for DB1017, DB244, DB249, DB103, DB60, DB935, and DB829 were based on analytical methods previously described for DB75 and DB820 (24, 30). HPLC Solvent A consisted of 15 mM ammonium formate, 30 mM formic acid in HPLC water, while Solvent B contained the same buffer system prepared in 4:1 acetonitrile: HPLC water. A gradient elution was used to resolve each diamidine, with starting conditions of 95% Solvent A: 5% Solvent B, which increased linearly over 10-15 min, followed by a 3-5 min re-equilibration to starting conditions. An Agilent Zorbax Bonus RP (New Castle, DE) column (2.1 X 50 mm, 3.5µm) was used for compound resolution. Compounds were detected using their maximum excitation and emission wavelengths (Table 4.3).

Mass Spectrometry Analysis of Diamidines. DB690, DB867, DB1057, and DB879 concentrations were determined by Liquid Chromatography/Mass Spectrometry (LC/MS). To verify results, analyses of DB103 and DB60 were conducted via HPLC with fluorescence detection as well as LC/MS. Depending on molecular weight of the test diamidine, either DB75 or DB103 was used as internal standard. Automated sample analysis was performed using Analyst software (version 1.4.1, Applied Biosystems, Foster City, CA). The Analyst controlled HPLC-MS/MS system consisted of two Shimadzu Scientific (Columbia, MD) solvent delivery pumps, a thermostated (6 °C) LEAP HTC autosampler (Carrboro, NC), and an Applied Biosystems API4000 triple quadrupole mass spectrometer. Reversed-phase gradient chromatography was used to elute the diamidines from an Aquasil (C18 5µm, 50 × 2.1 mm) analytical column (Torrance, CA) at a flow rate of 0.75 ml/min, following a 4µL

injection. Starting conditions for each injection were 95:5, water/methanol with 0.1% formic acid (vol/vol) in each. The relative amounts of water/methanol were held constant for 0.5 min while the column eluted to waste. After 0.5 min, the eluent was directed to the mass spectrometer and the relative amount of methanol increased linearly to 90% at 3 min post-injection. This amount of methanol was held for 0.5 min to wash the column. The column was re-equilibrated under the starting conditions for the final 0.5 min. Total run time was 4 min. The mass spectrometer was connected to the HPLC system by a TurboIonSpray interface. Nitrogen, from a Peak Scientific (Bedford, MA) nitrogen generator, was used as the curtain, nebulizer, and collision gases. User controlled voltages, gas pressures, and source temperature were optimized for the detection of the parent and product ions of the diamidines. All diamidines were analyzed in positive ion mode using multiple reaction monitoring.

D. Results

In vitro Antitrypanosomal Activity of Diamidines. Two series of diamidines were investigated – the diphenyl furan series, which includes DB75, and the aza analogs, which includes DB820 (Figures 4.1 and 4.2). Compounds in the diphenyl furan series had the greatest structural diversity, with modifications to the core structure, N-substitutions on the diamidines, and cyclic diamidines. Compounds in the aza series had less structural diversity, with modifications to the core structure only. All compounds were active in vitro, although activities against S427 *T. b. brucei* and STIB900 *T. b. rhodesiense* varied greatly (Tables 4.1 and 4.2).

The majority of the diamidines used in this study have two positive charges at physiological pH (pH 7.0), and lower pH values. pKa values for DB75, for example, have been determined to be around pH 11 (Table 4.1). Calculated LogD and calculated pKa values, obtained from ChemAxon MarvinSketch (http://www.chemaxon.com/demosite/marvin/index.html), are shown in Tables 4.1 and 4.2 for both series of compounds. ChemAxon's program calculated values consistent with measured values. This program was a better predictor than ACD LogD Suite v 4.5 (Advanced Chemistry Development, Inc, Toronto, CA). As seen in Tables 4.1 and 4.2, pKa values for all the diamidines are approximately 10 or greater, indicating that the compounds will be doubly charged at physiological pH. Most compounds, with the exception of DB244 and DB249, are hydrophilic at pH 7.4. DB244 and DB249 are both slightly lipophilic at this pH, likely due to the substitution of bulky cyclic groups off of the diamidines, which could mask the positive charges.

Diphenyl furans. Diamidines in the diphenyl furan series had IC_{50} values ranging between 2 and 260 nM against the S427 strain, a greater than 100-fold difference in activity (Table 4.1). The three compounds with unmodified diamidines (DB690, DB75, and DB1017) were the most potent. Changes in the bond positioning (DB690) or addition of functional groups to the core nucleus (DB1017) had only a modest 2-3 fold effect on activity compared to DB75. Compounds which had substituted amidines (DB244, DB249) had intermediate potency against S427 trypanosomes, with IC_{50} values between 100 – 150 nM. The cyclic diamidines, the imidazoline DB60 and the tetrahydropyrimidyl DB103, were the least potent compounds in this series against S427 trypanosomes. Against the STIB900 strain, the trend in activity for diphenyl furans was similar to that of the S427 strain, with 6 of the 8 compounds having the same rank order against both strains (Table 4.1). The only exception was DB103, which had an IC_{50} value of over 100 nM in the S427 strain, but was much more active against the STIB900 strain (IC_{50} value of 33 nM),

Aza analogs. All aza analogs had unmodified amidine moieties. Five of the six compounds were potent, with activities against S427 *T. b. brucei* varying only 10-fold (Table 4.2). DB867 was the most potent compound, with an IC_{50} value of 3.3 nM. The positioning of the nitrogen in the aromatic rings had only minor effects (DB867, DB820, and DB829). As with the diphenyl furans, there was little impact on activity when functional groups were added to the aromatic rings (DB935, DB1057). Between DB867 and DB1057, there is only a 10-fold difference in IC_{50} values. DB879, which has a methylene group inserted between the furan and phenyl rings, was much less potent than other compounds in this series. IC_{50} values for the compounds in this series against the STIB900 strain were similar for all compounds, and

in the same rank order. DB879 was about 2 fold more potent against STIB900 trypanosomes than against S427 trypanosomes, but overall was still not a very potent compound.

Thermal Melting Analysis of DNA binding. Increases in T_m values for compound-DNA oligomer and compound-polymer complexes are shown in Tables 4.1 and 4.2. The majority of the compounds in these two series were potent DNA binders and bound strongly to both polydA·polydT, which has multiple AT binding sites, and to the d(CGCGAATTCGCG) oligomer duplex, which has a single AT binding site for diamidines (Tables 4.1 and 4.2). Studies with a wide array of diamidines have shown that evaluation of increases in the melting temperature of DNA on addition of the compounds provides a rapid method for ranking diamidines according to their binding affinity (23).

All of the diphenyl furans are potent binders with ΔT_m values greater than 15°C for polydA·polydT and greater than 8°C for the d(CGCGAATTCGCG) oligomer duplex. The largest increases in the melting temperature were seen with DB244, DB249, DB103, and DB75 (Table 4.1), however, there was no correlation between activity of the diphenyl furans and DNA binding affinity ($r^2 = 0.09$). With the aza analogs (Table 4.2), there appeared to be a correlation between the activity and the increase in melting temperature with the polydA·polydT oligomer and the d(CGCGAATTCGCG) oligomer duplex ($r^2= 0.9$, slope -- 0.05 and 0.03 respectively), but this correlation was driven by the low ΔT_m values determined with DB879. When DB879 is removed from the series, no correlation exists (r^2 equal to 0.4 and 0.03 for the two oligomers respectively). DB879, the least potent compound investigated, had little effect on the melting temperature of the DNA complex (Table 4.2).

Intracellular Accumulation of Diamidines in S427 *T. b. brucei.* DB75 and DB820 were previously shown to accumulate to approximately 12 mM and 3 mM, respectively, after a 24

hour in vitro incubation with 7.5 μM of either compound (24). Since the compounds chosen for investigation have a wide range of in vitro activity, we chose the concentration of 7.5 μM to incubate with trypanosomes to compare to previous work. After investigating the accumulation of several of the diamidines, the pattern of intracellular concentration was similar to that reported previously (24). However, the toxicity of some of the compounds became an issue with the accumulation experiments (increasing cell death when incubated for longer than 8 hours), so investigations were limited to 8 h.

Due to poor fluorescence intensity, some compounds were analyzed and quantified by LC/MS instead of HPLC, which was used for the more fluorescent compounds. DB75, DB1017, DB244, DB249, DB103, DB60, DB935, DB820, and DB829 were analyzed using HPLC, while DB690, DB867, DB1057, and DB879 were analyzed by LC/MS.

Diphenyl furans. All of the diphenyl furans accumulated to high concentrations, ranging from peak concentrations of 1 mM to 30 mM (Figure 4.3) after 8 h incubation with 7.5μM. Three of the diphenyl furans (DB690, DB244, DB249) investigated accumulated to higher peak concentrations in trypanosomes than seen previously for DB75 (4.5 ± 0.3 mM at 8 h, Figure 4.3A). DB690, the most potent diphenyl furan investigated, as well as DB244 and DB249, with intermediate potency, accumulated to peak concentrations of approximately 30 mM (Figure 4.3A). The other diphenyl furans accumulated in trypanosomes to peak concentrations similar to that of DB75 (Figure 4.3B). Thus, there appears to be no trend linking potency of the diamidines and accumulation of the diphenyl furans in trypanosomes over time.

Aza analogs. Compounds in the aza analog series accumulated linearly over 8 h in trypanosomes for the most part (Figure 4.4), except for DB867, which accumulated in a non

linear manner. DB820 had a peak concentration of 1.0 ± 0.2 mM at 8 h. DB867, the most potent compound in this series (Table 4.2), which has the nitrogen in the pyrimidine ring moved *meta*- to the furan ring, accumulated to a concentration of 9.3 ± 2.0 mM after 8 h, which is significantly greater than the accumulation of DB820. DB935, which has a methyl substituent on the phenyl ring, accumulated to concentrations in trypanosomes 4-fold higher than DB820, despite being 3 fold less potent. All compounds in the aza analog series accumulated to higher concentrations than DB820, including DB879, the least potent diamidine investigated, as seen in Figure 4.4.

Fluorescence Spectra of Diamidines in Aqueous Solution. The fluorescence spectra of diamidines were determined for developing analysis methods, and for the fluorescence microscopy studies. Fluorescence of the diamidines varied in terms of intensity, and excitation (λ_{ex}) and emission (λ_{em}) wavelengths (Table 4.3).

Diphenyl furans. In the diphenyl furan series, four compounds had similar fluorescence spectra in water. DB75, DB1017, DB244, and DB249 had excitation maxima around 355 nm, with maximum emission around 455 nm. Of these compounds, DB249 had the highest emission intensity, and DB75 had the lowest emission intensity. Other structural modifications to DB75 tended to shift either the excitation wavelength by about 20 nm (DB690 or DB60), or the emission maximum by 20 nm (DB103) in either direction. In addition to shifting fluorescence spectra, some of the modifications also greatly decreased fluorescence intensity, as seen with DB690 (Table 4.3).

The fluorescence properties of select diphenyl furan diamidines were also investigated in the presence of AT rich DNA. The fluorescence of DB690, which is only weakly fluorescent in solution, increased almost 300% in the presence of DNA, while the

fluorescence of DB75 decreased 17% in the presence of DNA (Table 4.3). The fluorescence intensity of DB60 also decreased by 23% when bound to DNA.

Aza analogs. In the aza series, only DB820 and DB935 had similar excitation and emission spectra, with a maximum excitation of about 360 nm and emission around 460 nm. The spectra of other compounds were shifted in either excitation or emission wavelength or both. Placing the nitrogen in the pyrimidine ring *ortho-* to the amidine (DB867) opposed to *meta-* to the amidine (DB820), shifted the excitation maximum by 10 nm, and the emission maximum was also shifted by 20 nm. This modification also decreased the fluorescence intensity by approximately 60 fold. However, having two nitrogens – one in each pyrimidine ring (DB867) increased the fluorescence intensity two-fold over that of DB820, while shifting the maximum emission wavelength down by 20 nm. Addition of either an alcohol group to the phenyl ring or addition of a methylene linker between the phenyl and furan ring decreased the fluorescence intensity dramatically (Table 4.3).

The fluorescence of select diamidines was investigated in the presence of AT rich DNA. As with DB690 in the diphenyl furan series, fluorescence of the weakly fluorescent DB867 is greatly enhanced (almost 400%) with increasing concentrations of DNA (Table 4.3). The very fluorescent DB820 and DB829 exhibit decreased fluorescence when bound to DNA (50% and 86%, respectively).

Intracellular Distribution of Diamidines in S427 *T. b. brucei*. The majority of the compounds in both series distributed to three organelles in the trypanosomes: the kinetoplast, the nucleus, and the acidocalcisomes (See next section). A concentration of 500 nM was chosen for microscopy studies for several reasons, including prevention of fluorescence signal saturation of the compounds, and because of the toxicity of some

compounds at higher concentrations. For some compounds that were less fluorescent, distribution at 5 μM was also investigated, but no difference in the fluorescence intensity within trypanosomes was identified.

DB75 localization to the acidocalcisome. After two hour incubation in vitro with S427 trypanosomes, DB75 accumulated in the nucleus, kinetoplast and acidocalcisomes (Figure 4.5, first column). However, when ammonium chloride or monensin were used as a pretreatment before DB75 was added, accumulation in the acidocalcisomes was not seen (Figure 4.5, columns 2 and 3). A 60 min pretreatment of ammonium chloride was needed to prevent DB75 accumulation in acidocalcisomes, although partial inhibition of acidocalcisomes localization was seen after 30 min. The sodium ionophore monensin, on the other hand, only needed to be incubated with trypanosomes for 15 min before DB75 was added to abolish acidocalcisome accumulation. Partial inhibition of accumulation was seen after 5 min. Additionally, monensin prevented acridine orange fluorescence in the acidocalcisomes of trypanosomes after only 15 min (data not shown). We were unable to use fixation and colocalization techniques due to issues with redistribution of DB75 or loss of fluorescence (30).

Diphenyl furans. DB75, accumulated in the nucleus, kinetoplast and acidocalcisomes after 8 hrs of in vitro incubation (Figure 4.6). There were four compounds in the diphenyl furan series that had similar fluorescence spectra and relatively similar fluorescence intensity – DB75,DB1017, DB244, and DB249. Compounds with similar fluorescence properties can be compared directly, but it must be considered that each compound may behave differently when accumulated in cells (i.e. fluorescence could be shifted, enhanced or quenched upon sequestration in organelles, and this may be different for each compound). Like DB75,

DB1017 appeared to be localized to the kinetoplast, nucleus and acidocalcisomes (based on comparison of localization between DB75 and DB1017). The color shift in the acidocalcisomes with this compound is to more of an orange color, compared to the yellow color seen in DB75 stained acidocalcisomes. DB244 and DB249 were both found only in the nucleus and kinetoplast (Figure 4.6).

For the rest of the diphenyl furans, the excitation and/or emission wavelengths were shifted slightly, and no direct comparisons can be made as to their relative fluorescence intensities. DB690 appeared to accumulate only in the kinetoplast as shown in Figure 4.6. However, due to the weak innate fluorescence of DB690 (Table 4.3), it is possible that there is some low level fluorescence in other organelles in the trypanosomes which we were unable to observe. Additionally, the peak excitation of DB690 is below the limit of the UV2A cube, and we are likely not seeing the compound excited at its maximal excitation wavelength. The imidazoline DB60 was localized to the kinetoplast, nucleus and acidocalcisomes. DB103 accumulated only in the nucleus and the kinetoplast (Figure 4.6).

Aza analogs. DB820, as shown previously, was localized to the nucleus, kinetoplast, and faintly in the acidocalcisomes (Figure 4.7). DB935, the only compound with similar excitation and emission wavelengths to DB820, was localized to the nucleus and kinetoplast. The most potent analog, DB867 appeared in the nucleus, kinetoplast and acidocalcisomes (Figure 4.7). Despite being weakly fluorescent in solution, the compound exhibited intense fluorescence in trypanosomes, which may be related to an increase in fluorescence when bound to DNA (Table 4.3). DB1057 was found in the nucleus and kinetoplast, while DB829 was found in the nucleus, kinetoplast and acidocalcisomes. DB879, the least potent of the DB820 analogs was located very faintly in the kinetoplast. The compound is weakly

fluorescent, which may affect the appearance of fluorescence seen in the trypanosome, although DB879 concentrates in trypanosomes to levels comparable to that of the other aza analogs.

E. Discussion

Diphenyl furan and aza diphenyl furan diamidines represent two potent classes of antitrypanosomal compounds. The compounds shown here had a greater than 150 fold range of in vitro activity, and some had different organelle distribution within the cells. However, despite the wide range of activity and distribution and, for the most part, similar DNA binding properties, the compounds accumulated to similar concentrations in the cells, with only a few exceptions.

Eight compounds in the diphenyl furan series were investigated. The substituted and cyclic diamidines were the least potent compounds in this series, and the fact that modification to the diamidine affects their potency reinforces the importance of the moiety in the potential mechanism of action of these compounds. Other modifications of DB75 had little effect on the IC_{50} value, only changing potency by approximately 2 fold.

In the aza analog series, the only modification to have a major effect on potency was the methylene linker in DB879, which also decreased DNA binding, likely due to the change in curvature of the molecule. This illustrates the importance of a threshold DNA binding activity necessary for antitrypanosomal activity of these compounds.

DNA binding for the two series of compounds, as determined by measuring the ΔT_m in the presence of compound, was similar for most of the compounds. With the exception of DB879, all of the compounds tested increased the melting temperature of a polydA·polydT complex by greater than 15ºC. With DB879, the methylene linker appears to inhibit DNA binding. Surprisingly, differing effects were seen when fluorescence of select diamidines was measured when the compounds were bound to DNA. The least fluorescent analogs, DB690 and DB867, exhibited increased fluorescence when bound to AT rich DNA, while

more fluorescent analogs had decreased fluorescence when bound to DNA. The DNA used for this experiment was chosen because of its high AT content. However, conclusions about changes in fluorescence in the trypanosome organelles can not be made until measured with isolated kinetoplast and nuclear DNA.

We have previously proposed (34) that interaction with DNA alone was insufficient to generate biological activity. In one possible mechanism for biological activity of the dications, however, DNA binding would be an initial requirement but specific secondary effects, which occur subsequent to binding, would be required for activity. Such effects could be topological changes induced in DNA, as a result of binding that cause errors in DNA structure with resulting instability and eventual destruction. It is also possible that binding could result in direct inhibition of DNA targeted enzymes or control proteins, which could also lead to cell death. These secondary effects could vary significantly among compounds with similar binding affinities for DNA and could account for the different activities of compounds with similar uptake and DNA affinity. The set of compounds in this paper that have similar DNA binding and uptake would make an excellent test set for testing the possible influence of secondary effects, after DNA binding, on biological activity.

Changes to the structure of the compounds also resulted in changes in the accumulation of the compounds in trypanosomes. Surprisingly, some of the more potent compounds, including DB75 and DB820, accumulated to lesser extent than some less active compounds, such as DB244, and DB249. Additionally, in the aza analog series, all of the compounds investigated accumulated to higher concentrations in trypanosomes than the very potent compound DB820 (Figure 4.4).

There also does not appear to be any correlation between which organelles the compounds are distribute to and overall accumulation in trypanosomes. For example, DB820, one of the most potent compounds examined, had the lowest accumulation in trypanosomes. Of the three compounds that accumulate to the highest concentrations in trypanosomes, one appears to be localized to the kinetoplast only (DB690), while the other two are localized to the nucleus and kinetoplast (DB244 and DB249). DB690 was the most potent of the compounds tested, while DB244 and DB249 had intermediate IC_{50} values, yet all three accumulated to similar concentrations in trypanosomes. However, as stated previously, DB690 is a weakly fluorescent compound with a peak excitation wavelength below the limit of the UV2A cube used for fluorescence microscopy. It is thus possible that we are not seeing the true distribution of DB690 in trypanosomes, even accounting for the enhancement of DB690 fluorescence when bound to AT rich DNA (Table 4.3). This indicates that neither concentrations nor distribution in trypanosomes can be predicted from in vitro activity. Additionally, the site of localization in trypanosomes cannot be predicted from the accumulation in the cells, or vice versa.

It is likely from these series of diamidines that DNA binding, in either the nucleus or kinetoplast, is important, especially considering that for the most part, all of these compounds are excellent DNA binders. Further studies are needed to determine relative amounts of diamidines in each organelles, as well as relative affinity for DNA associated proteins found in either, or both organelles. Only then can organelle specific mechanisms of action be determined. Additionally, changes in fluorescence intensity and/or shifts in excitation or emission wavelengths may occur when compounds bind DNA, as shown in Table 4.3. Although the DNA used for that investigation was AT rich, to be certain of relative

fluorescence changes in the nucleus or kinetoplast, DNA from both organelles must be isolated and binding and fluorescence changes measured. With the dramatic changes seen here, these measurements should be routinely determined in the future in order to extrapolate from DNA binding to distribution studies.

Tools used for investigating transport and uptake of compounds in trypanosomes include rapid transport experiments with various inhibitors that prevent uptake via particular transporters. Transport of diamidines has been attributed to uptake via the P2 transporter (5, 8, 22, 29), and in the case of pentamidine, the HAPT1 and LAPT1 are also involved in uptake and accumulation (4). Lanteri et al. were able to inhibit uptake of DB75 over 30 sec with inhibitors of the P2 transporter such as adenosine, adenine, pentamidine, and diminazene (21), indicating that it is likely a substrate of the P2 transporter. However, transport studies with all of the compounds investigated here have not been implemented, and further investigation is needed to determine the impact of transporters on long term accumulation. The high levels of DB690, DB244, and DB249 in trypanosomes may indicate that others transporters or transport processes are involved in the uptake of this compound.

The effects of endogenous substrates of the transporters, such as adenosine, can be assayed during in vitro antitrypanosomal activity experiments to determine if the presence of other substrates affects potency (i.e. increases the IC_{50} value) of the test compounds (13). When an IC_{50} value was determined for DB75 in the presence of 1 mM adenosine (a substrate of the P1 and P2 transporters), we found that the IC_{50} value increased 10-fold over controls without adenosine (data not shown). However, there was no effect seen in the presence of inosine, a substrate for the P1 transporter. This phenomenon has also been shown with the adenosine antimetabolite tubercidin in trypanosomes, where addition of 1

mM adenosine or inosine decreased the antitrypanosomal IC_{50} value of tubercidin 4-5 fold (13). The decrease in potency of DB75 in the presence of excess adenosine is likely due to less DB75 getting into trypanosomes, but this should be further evaluated over time to determine if there is a significant reduction in the amount of DB75 accumulating in the trypanosomes.

Additionally, the impact of increasing the lipophilicity of compounds on the transport properties, as with DB244 and DB249, should be further investigated. It is possible that other transporters or uptake processes involved in the accumulation of the more lipophilic compounds. Is it possible that these compounds are accumulated by both passive diffusion and active transport? Both DB244 and DB249, according to calculations performed using ChemAxon MarvinSketch, are lipophilic at physiological pH 7.4, and could potentially diffuse across trypanosome membranes.

We also attempted to investigate the accumulation and distribution of another compound in the diphenyl furan series, DB569. DB569 is an N-phenyl substituted diamidine. In cancer cells, DB569 was found localized to the mitochondria instead of the nucleus (20). However, this compound was rapidly toxic to trypanosomes, inducing cell death in 1 h. At lower concentrations, the substituted diamidine appeared to be widely distributed throughout trypanosomes (data not shown). Increased lipophilicity could be one reason that DB569 rapidly kills trypanosomes, due to high levels accumulating in trypanosomes. The pKa of DB569 has been determined to occur at pH 6.9 (average, data not shown), indicating that at the pH of the media (pH 7.4), over half of the drug is unionized, compared to less than 1% unionized with DB75, which has a pKa around 11. The LogD of DB569 at pH 7.4 has been calculated to be 5.1 using ChemAxon MarvinSketch, indicating

that the substituted diamidine is highly lipophilic at this pH and would likely diffuse across cell membranes more rapidly than unsubstituted diamidines. DB569 is an interesting diamidine, as it has very different physicochemical properties than other diamidines we have examined. Additionally, due to its rapid killing of trypanosomes, it may have different mechanisms of action. It is clear that more than one approach is needed to fully characterize the processes of transport and accumulation of diamidines in trypanosomes. One interesting avenue would be to investigate the long term accumulation of diamidines in trypanosomes that lack the P2 transporter.

All of the compounds investigated in both series of diamidines were found in the nucleus or the kinetoplast, or both. A few of the compounds, of varying potency, also accumulated in the acidocalcisomes. Due to previous evidence to support DNA binding as a mechanism of action, primarily studied with pentamidine (27, 28), our original hypothesis when we began working with DB75 and DB820, was that their mechanism of action would be related to DNA binding as well. It is still not understood what impact the acidocalcisomes could have on the mechanism of action. It may be that even in this closely related group of compounds, there could be potentially two or three mechanisms of action, involving DNA in the nucleus and kinetoplast, as well as some mechanism in the acidocalcisomes. As dicationic molecules, the diamidines may accumulate in the low pH acidocalcisomes and interfere with homeostasis mechanisms in the trypanosomes.

Many questions still remain about the mechanisms of action in these compounds. The lack of correlation between accumulation and activity of the compounds is intriguing. Why are some of the less active compounds accumulating to levels much higher than the

more active compounds? There is also likely to be a time dependency to the amount of drug that is necessary to kill trypanosomes.

Investigations with DB569, DB75 and DB820 indicate that only about 30 minutes to 1 hour of drug treatment is necessary to cause trypanosome death. With DB75 and DB820, death occurred 24-48 h after washing away drug (data not shown), but with DB569, death occurred within 1 hour, which may indicate a different mechanism of action than that of DB75 and DB820. DB569 also has been shown to cause apoptosis in *T. cruzi*, to a greater extent than DB75 (10). However, no indication of the time in which this process needs to take place was indicated. In vivo, DB569 resulted in increased survival of mice infected with *T. cruzi*, and decreased cardiac parasitism, but was unable to clear parasitemia (11). Since we were unable to investigate the accumulation of DB569 in our trypanosomes, we cannot compare it completely to other substituted diamidines such as DB244 and DB249. However, the differences in killing seen between DB569 and other compounds in this series are very intriguing and require further investigation, especially since it would appear to be not very potent on a typical in vitro screen for antitrypanosomal activity, and in light of its different physicochemical properties.

In summary, the accumulation and distribution of a diphenyl furan or an aza analog in trypanosomes is not indicative of its antitrypanosomal activity. In actuality, there may be a minimum in vitro accumulation of a diamidine in trypanosomes necessary for killing. From this study, it seems that a minimum accumulation concentration of 1 mM in trypanosomes is necessary for this type of compound – DB820, the compound that accumulated the least in trypanosomes, was also one of the most potent compounds. Whether this 'minimum accumulation level' would hold true for other series of diamidines remains to be investigated.

The overall accumulation depends on a variety of factors, including which transporters play a role in accumulation and the relative affinity for the transporters as well. The factors that affect transport, accumulation, and ultimately mechanism of action will be further investigated using this series of diamidines, as well as other, more structurally diverse diamidines.

Table 4.1: in vitro activity and DNA binding (ΔT_m)of select diphenyl furans

Compound	pKa	cLogD*	IC_{50} value (nM)		ΔT_m	ΔT_m
			S427	STIB 900	(polydA.polydT)	$(dCGCGAATTCGCG)_2$
DB690	10.8, 11.6	-2.8	2.1	1.7	17.1	10.4
DB75	10.4, 11.8	> -2.0	7	3.2	26.2	8.9
DB1017	10.5, 11.3	-1.2	14.8	8.0	15.2	8.2
DB244	10.3, 11.1	0.2	100	60	27	16
DB249	10.3, 11.1	1.0	121	68	27	16.8
DB60	8.8, 9.5	-0.4	251	245	19.3	11.0
DB103	9.5, 10.2	-1.3	260	33	27	15.1

*cLogD values (at pH 7.4) and pKa values were calculated using ChemAxon MarvinSketch (http://www.chemaxon.com/demosite/marvin/index.html).

[1]DB75, DB1017 pKa and LogD values were measured by Dr. J. Saulter (UV spectrophotometric method) .

Table 4.2: in vitro activity and DNA binding (ΔT_m) of select aza analogs

Compound	pKa	cLogD*	IC_{50} value (nM)		ΔT_m	ΔT_m
			S427	STIB 900	(polydA.polydT)	$(dCGCGAATTCGCG)_2$
DB867	8.5, 11.1	-2.8	3.3	2.1	23.3	10.4
DB820	10.5, 11.8	-3.8	3.4	6.5	20.0	11.2
DB935	9.9, 11.3	-4.5	10	9.7	16.7	10.6
DB829	9.6, 10.3	-3.3	13	19	16.0	8.4
DB1057	7.4, 11.6	-2.7	32	32	16.4	10.4
DB879	9.9, 11.6	-3.5	314	147	3.1	0.4

*cLogD values (at pH 7.4) and pKa values were calculated using ChemAxon MarvinSketch (http://www.chemaxon.com/demosite/marvin/index.html).

Table 4.3: Fluorescence properties of aqueous solutions (500 nM) of diphenyl furan and aza analogs and Change in fluorescence of select analogs when bound to DNA

Compound	λ_{ex} max	λ_{em} max	Intensity at Max	% Change in Fluorescence when Bound to DNA[1]
DB690	323	452	51	↑ 270%
DB75	356	458	464	↓ 17%
DB1017	354	455	840	ND[2]
DB244	356	450	625	ND
DB249	356	453	920	ND
DB60	373	458	630	↓ 23%
DB103	352	437	860	ND
DB867	370	481	51	↑ 360%
DB820	359	455	318	↓ 50%
DB935	358	461	330	ND
DB829	359	433	701	↓ 86%
DB1057	352	470	16	ND
DB879	337	435	17	ND

[1]Change in fluorescence of compounds determined at a concentration of 1 μM
[2]ND: Not Determined

Figure 4.1. Structures of diphenyl furan diamidines, presented in order of IC_{50} values against *T. b. brucei* S427 trypanosomes. DB75, which is bolded, represents the prototypical diamidine in this series. All other compounds are modifications of DB75.

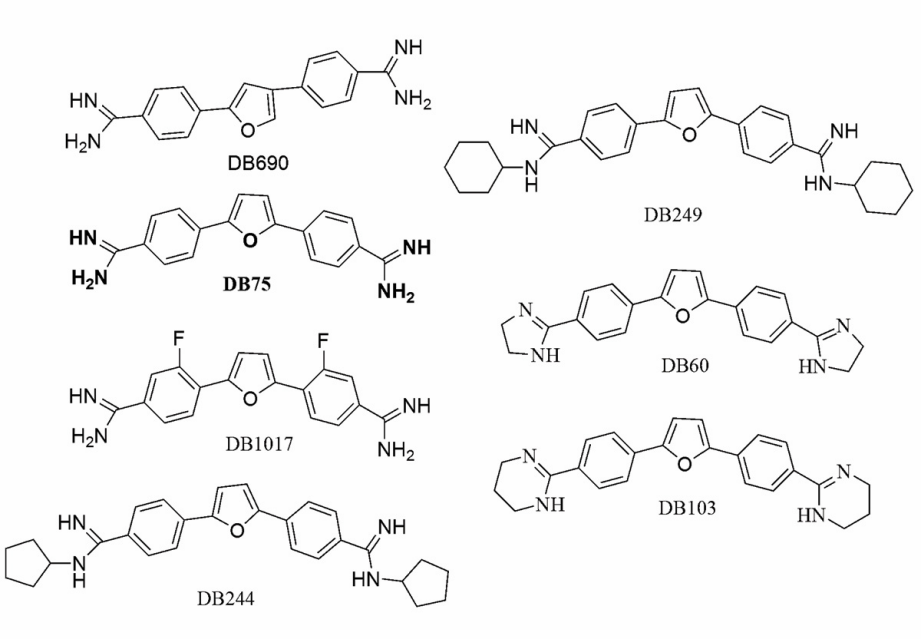


Figure 4.2. Structures of the aza analog diamidines, presented in order of IC_{50} values against *T. b. brucei* S427 trypanosomes. DB820, which is in bold type, represents the prototypical diamidine in this series. All other compounds are modifications of DB820.

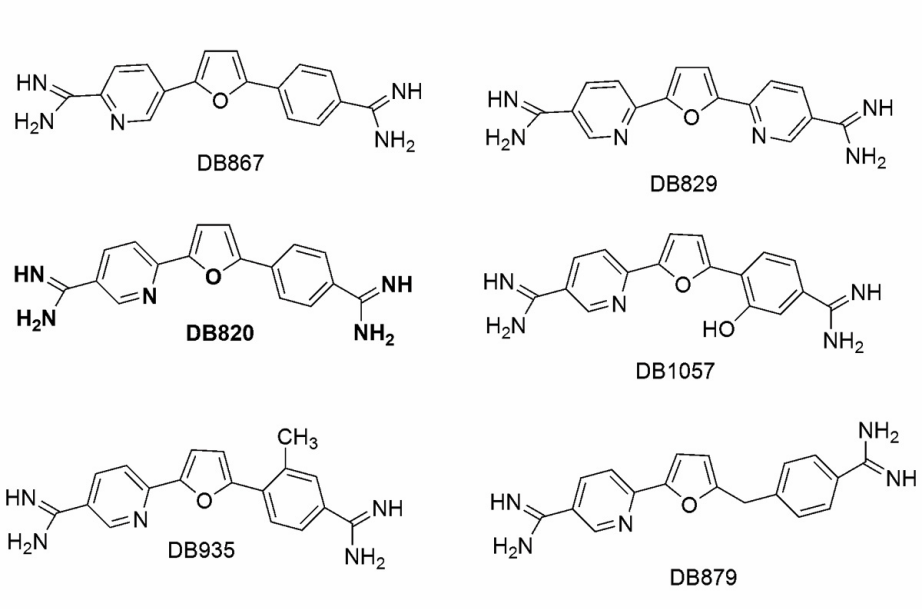


Figure 4.3. In vitro accumulation of diphenyl furans (7.5 µM) over 8h. Shown in Figure 4.3A is the accumulation of diphenyl furans to concentrations much higher than DB75. Figure 4.3B depicts accumulation at lower millimolar levels over 8h. Accumulation is presented as concentration ± SE.

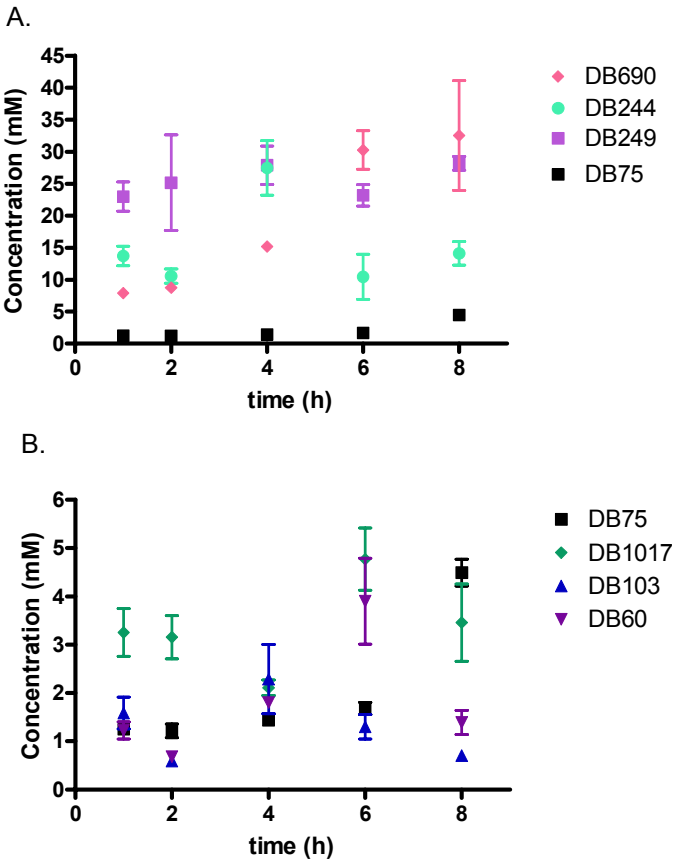


Figure 4.4. In vitro accumulation of Aza analogues (7.5µM) over 8 h. Data is presented as concentration ± SE.

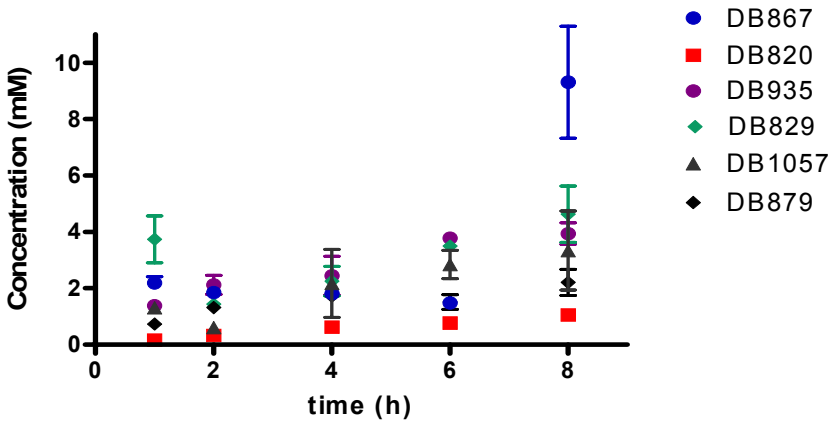


Figure 4.5: In vitro distribution of DB75 (7.5 μM) after pretreatment with either ammonium chloride (20 mM) and monensin (5 μM).

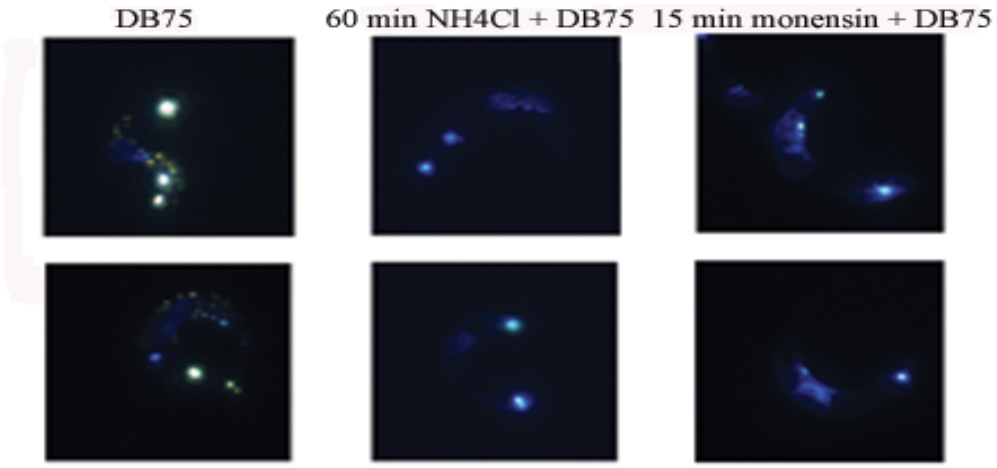


Figure 4.6. Fluorescence microscopy of the diphenyl furans (listed in order of IC_{50} values) at 8 hours following incubation with 500 nM of each compound. Images on the left depict phase contrast images of trypanosomes, while on right are fluorescence images of the same trypanosome.

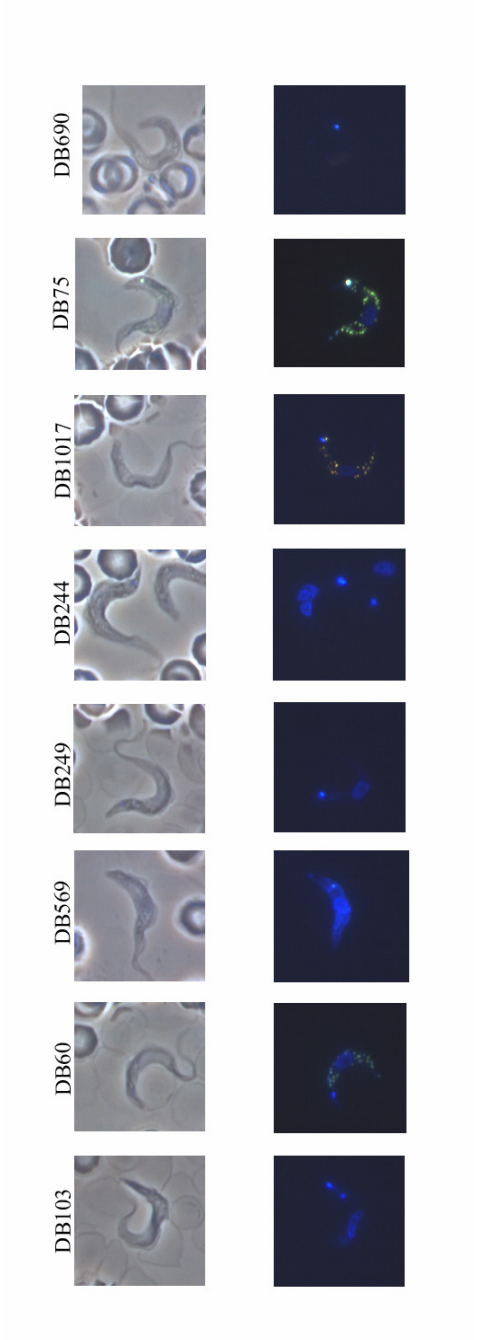
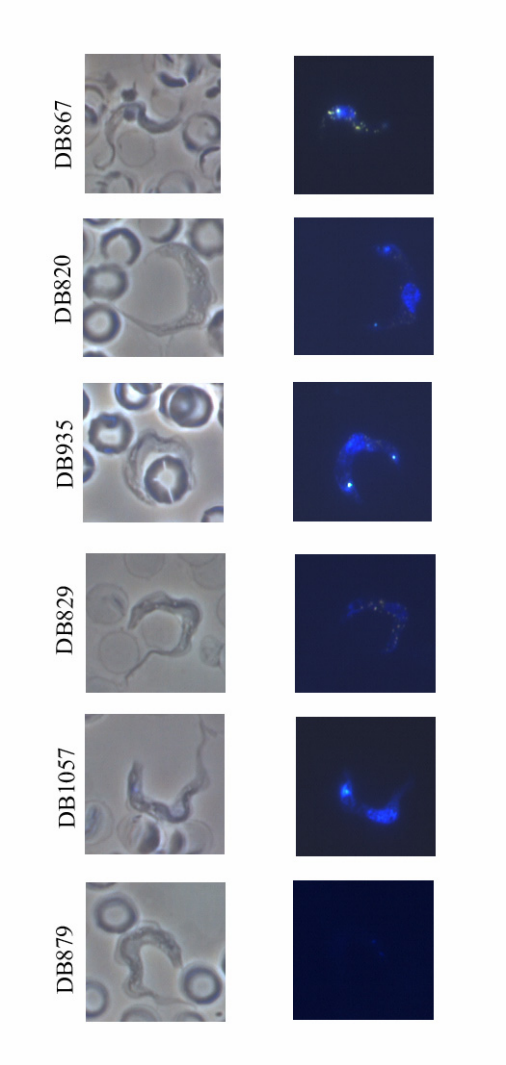


Figure 4.7. Fluorescence microscopy of the aza analogues (listed in order of IC_{50} values) at 8 hours following incubation with 500 nM of each compound. Images on the left depict phase contrast images of trypanosomes, while on right are fluorescence images of the same trypanosome.



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CHAPTER 5

IN VITRO ACTIVITY AND INTRACELLULAR DISTRIBUTION OF BENZOFURAN AND BENZIMIDAZOLE DIAMIDINES IN AFRICAN TRYPANOSOMES

A. Abstract

Only four treatments exist for human African trypanosomiasis, and there is a great need for new therapies. All of the currently used drugs are associated with problems including toxicity, cost, the need for parenteral administration, and increasing reports of treatment failure and drug resistance. Pafuramidine, or DB289, the methoxime prodrug of furamidine (DB75), is the only new drug developed for the treatment of trypanosomiasis in recent years. Pafuramidine is currently in Phase III clinical trials in Africa. However, pafuramidine is ineffective against late stage trypanosomiasis, so drug development efforts continue. A library of more than 2000 diamidine analogs has been synthesized with varying anti-trypanosomal activity in vitro and in vivo. It has long been thought that DNA binding plays an important role in the mechanism of action of diamidines. Here in vitro activity and distribution for two series of diamidines is presented. Both series of compounds have a wide range of in vitro activity. The benzimidazoles show the typical distribution pattern shown to be associated with related diamidines, localizing to the nucleus and kinetoplast. Compounds in the benzofuran series do not appear to extensively localize to the DNA containing organelles, but instead are found in cytoplasmic organelles believed to be the acidocalcisomes. One benzofuran, 150OXD049, was further investigated in vitro. Despite only a 200 fold concentration in trypanosomes over plasma, 150OXD049 was able to cure trypanosome infection in mice. The benzofuran series of diamidines are a new class of compounds with potentially different mechanisms of action than previously studied diamidines.

B. Introduction

There is a great need for new treatments of parasitic diseases such as human African trypanosomiasis. Currently only four drugs are available for treatment of trypanosomiasis – pentamidine, suramin, melarsoprol, and eflornithine (11). Pentamidine and suramin are hydrophilic compounds which can only be used to treat the first stage of the disease, when trypanosomes are confined to the bloodstream. For treatment of the late stage of trypanosomiasis, melarsoprol and eflornithine are used (6, 7, 11). In addition to being stage specific, treatment is also parasite specific. There are two forms of trypanosomiasis caused by morphologically identical parasites – *Trypanosoma brucei rhodesiense and T. brucei gambiense.* The Gambian form of the infection is chronic, while the Rhodesian form is more acute and virulent (1). Pentamidine is used for *T. b. gambiense* infections, while suramin is used to treat early stage *T. b. rhodesiense* infections. While melarsoprol can be used to treat the late stage of infection caused by either parasite, eflornithine is only effective against the Gambian form of the infection (7).

In addition to the limited treatment options, all currently used therapies are associated with problems including toxicity, treatment failures and drug resistance, cost of production, and the need for parenteral administration (6, 7). The prodrug pafuramidine, or DB289, is the only compound to be developed for trypanosomiasis in recent years. DB289 is the prodrug of the active diamidine, DB75, which has been shown to have excellent antitrypanosomal activity in vitro and in vivo (9). However, DB289 is not effective against late stage trypanosomiasis, and therefore it is necessary to continue investigating diamidines to find compounds that will be effective against the late stage of the infection. A library of diamidines and prodrugs has been synthesized, which consists of many structurally diverse

diamidines, including diphenyl furans, benzofurans, benzimidazoles, and terphenyl diamidines with varying activity against trypanosomes (3-5).

The mechanism of action of diamidines is poorly understood. Evidence exists for the mechanism of diamidine action being DNA binding and topoisomerase inhibition, inhibition of protease inhibitors, or inhibition of polyamine metabolism (13-15). Although much work on mechanism of action has been done with pentamidine, it is unknown if the non fluorescent compound actually is located at the hypothesized sites of action. Fluorescent analogues of pentamidine have already been useful for investigating distribution inside trypanosomes with the hope of discovering mechanism of action. With a large number of compounds available it is possible to investigate the distribution of many compounds to determine whether different mechanisms occur with different classes of diamidines.

We have previously provided evidence for the in vitro activity, and accumulation and intracellular distribution of a series of fluorescent diaryl furan diamidines in trypanosomes (9), presented in Chapter 2 and 4. Here we have expanded those investigations to other series of compounds in the available diamidine library. Approximately 100 compounds from different classes were screened (Table 5.1). Two series of compounds are presented here. Compounds were investigated for fluorescence properties, in vitro activity, and intracellular distribution in trypanosomes. In these two series, a wide distribution of in vitro activity and intracellular distribution was found. One compound which showed a unique distribution in trypanosomes, 150OXD049, was further investigated in vivo to determine accumulation, distribution and activity in an infected mouse model of trypanosomiasis.

C. Methods and Materials

Materials. All materials and reagents were obtained from Fisher Scientific or Sigma unless otherwise stated.

Trypanosome Culture. S427 *T. b. brucei* trypanosomes were grown in vitro in CBMEM media as previously described (9). Trypanosomes were grown in 24 well plates (Corning Costar), and serially passaged twice a week.

Diamidines. Diamidine compounds were obtained from the Tidwell Laboratory at the University of North Carolina (benzofurans) and the Boykin Laboratory at Georgia State University (benzimidazoles). Benzimidazoles were synthesized as previously described (5, 8, 10). Benzofuran synthesis was as described (Bakanov, S, in preparation). These compounds are covered under the US patent application 20050197378.

Fluorescence Spectra of Diamidines in Aqueous Solution. Fluorescence scans to determine excitation and emission maxima were performed using a Perkin Elmer Luminescence Spectrophotometer (LS 50B) using FL Winlab Version 4.00.02 software (Perkin Elmer, Wellesley, MA). Compounds were prepared as 500 nM solutions in distilled water. One ml of each solution was transferred to a disposable cuvette (Fisher Scientific) and the fluorescence spectrum was determined. Fluorescence intensity measurements were then determined using the λ_{ex} and λ_{em} maxima.

In vitro Antitrypanosomal Activity. To determine IC_{50} values, *T. b. brucei* S427 trypanosomes (2×10^4 trypanosomes/ml) were cultured at 37 ºC and 5% CO_2 with serial dilutions of the diamidines for 72 h using a method previously described (12). Alamar Blue (10% vol/vol) was added after 70 h incubation with diamidines. At 72h, fluorescence was determined at λ_{ex}: 544 nM, and λ_{em}: 588 nm, using a PolarStar fluorescence plate reader

(BMG, Durham, NC). The fluorescence emission derived from each drug concentration was normalized to fluorescence of the untreated control trypanosomes. The background fluorescence (determined in wells in which 10% Alamar Blue was added to CBMEM media) was subtracted from total fluorescence values. IC_{50} values were determined using the Hill Equation (Graphpad Prism, San Diego, CA), and are the average of at least 3 determinations.

In vitro Distribution of Diamidines in S427 Trypanosomes. S427 trypanosomes were cultured in 24 well plates (Corning Costar, Fisher Scientific) at a starting concentration of 10^6 trypanosomes/ml. Diamidines were added to each well to a final concentration of 500 nM. Trypanosomes were incubated with the diamidines for 8 h at 37ºC, and then trypanosomes were examined under a light microscope for motility and viability. Trypanosomes were washed twice in fresh medium (1 ml) which contained no diamidine and resuspended in 20 μL of freshly isolated mouse blood. A small drop of blood was placed on a microscope slide and thin films were prepared. Thin films were used to reduce the photo bleaching that occurs when viewing live trypanosomes under the UV-fluorescence cube. No differences in intracellular distribution of fluorescence were seen when live trypanosomes were viewed under fluorescent light. Thin films were examined using Nikon Microphot FXA (Garden City, NJ) with a 60 DM x 1.4 NA objective lens, a mercury lamp, and a QImaging Micropublisher 3.3 CCD Digital Camera (QImaging Corporation, Surrey, BC, CA). QCapture version 3.0 imaging software was used to capture images (QImaging). The microscope was equipped with a Nikon UV2A cube that limits excitation wavelengths to 330-380 nm and emission wavelengths to ≥ 420 nm. Before viewing, thin films were mounted with a drop of glycerol.

In vivo Accumulation of 150OXD049 in Plasma and Trypanosomes. Male Swiss Webster mice (25-35 g) were infected with S427 trypanosomes (10^5 trypanosomes IP). All animal experiments adhered to guidelines outlined by the University of North Carolina-Chapel Hill Institutional Animal Care and Use Committee (IACUC). On the third day post infection, parasitemia was determined with a drop of blood from the tail vein. Infected mice were treated with 15 μmol/kg 150OXD049 administered intraperitoneally (IP). At selected times after treatment, mice were euthanized by CO_2 asphyxiation, and blood was collected by cardiac puncture into two lithium heparin coated microtainer tubes (BD Biosciences). As previously described (9), one tube was used to isolate trypanosomes from whole blood, while the other was used to determine plasma concentrations of DB75. Briefly, trypanosomes were isolated by mixing whole blood with a Percoll solution (100%) containing 0.25 M sucrose and 0.1 M glucose (pH 7.4). This mixture was centrifuged for 35 min at 13200 rpm (16100 g) in an Eppendorf 5415R microcentrifuge. Trypanosomes were isolated from suspension above the red blood cell layer, and were washed twice in 1 ml of phosphate saline glucose buffer, pH 7.4, counted and 150OXD049 was extracted with four volumes of 8:1 methanol: 1.0 N HCl (vol/vol). Half of each sample was evaporated in a Zymark Turbovap-LV (Hopkinton, MA) under nitrogen gas (7 psi) and reconstituted in HPLC solvent A. The remainder of the sample was retained for future analysis if needed. Plasma was isolated by centrifuging blood at 3000 rpm for 10 min to pellet red blood cells, and extracted as described above.

In vivo Distribution of 150OXD049 in Trypanosomes. Thin films were prepared from a drop of blood at each time point where trypanosome and plasma concentrations were determined. Thin films were mounted with a drop of glycerol, and examined using a Nikon

Microphot FXA microscope (Garden City, NJ) as before, but with an Optronics DEI 750 CCD camera (Goleta, CA). The UV2A cube was used to visualize 150OXD049 in trypanosomes.

HPLC Analysis of 150OXD049. Analytical methods for 150OXD049 were similar to those used for DB75 (Chapters 2-4). The solvent system consisted of HPLC solvent A (30 mM ammonium formate, 60 mM formic acid at pH 3.0) in 100% HPLC grade water, and HPLC solvent B (80% acetonitrile, 20% ammonium formate/formic acid buffer). A gradient was used to elute 150OXD049, with starting conditions of 95% solvent A/5% solvent B. Over 10 min, the % solvent B was increased linearly, followed by a 6 min re-equilibration to starting conditions.

An Agilent 1100 series HPLC equipped with a fluorescence detector (Agilent Technologies) was used for analytical procedures. DB75 and DB820 were eluted on a 3.5 μm Bonus – RP (Agilent Technologies) 2.1 x 50 mm column with a flow rate of 0.35 ml/min. The column was maintained at 25 ºC throughout the analytical method. Injection volume for each sample was 5 μl. The wavelengths used for fluorescence detection of 150OXD049 were λ_{ex} = 321 nm and λ_{em} = 425 nm. Compounds were quantified by comparing to standards injected during each analytical run. The standards were used to determine the concentration of 150OXD049 in plasma and in trypanosomes. Standards were linear between 5 nM and 1 μM. Concentrations were expressed as μM ± SE for plasma and trypanosomes.

D. Results

In vitro Antitrypanosomal Activity. A wide range of IC_{50} values was seen in the benzofuran series (Table 5.2). The compounds ranged from the very potent 150XD049, an unsubstituted diamidine with an IC_{50} value of 11 nM, to the isopropyl substituted 7SAB006, which has an IC_{50} value greater than 3 μM. 150XD049 was almost 300 fold more potent than 7SAB006. Similar to the effects seen in Chapter 4, substituting benzofurans also had a profound effect on the IC_{50} values. Surprisingly, differing effects were seen for the imidazoline benzofurans. 6SAB042 is almost 40 times more potent than 7SAB085; the only difference between the compounds was the $–OCH_3$ substituent on the benzofuran ring (Table 5.2).

The range of IC_{50} values in the benzimidazole series was similar (approximately 250 fold). The most potent benzimidazole, DB818, had an IC_{50} value of 0.9 nM, as seen in Table 5.3. The imidazoline version of this compound, DB819, was only 4 fold less potent, with an IC_{50} value of 3.6 nM. With an IC_{50} value of less than 5 nM, DB819 is still a very potent diamidine. This contrasts with the diphenyl furan series in Chapter 4, where the imidazoline substituted compound was 30 fold less potent than the unsubstituted diamidine. The two least potent compounds in this series were compounds that had pyrimidine rings incorporated into their structure: DB915 and DB920. DB915 was still potent with an IC_{50} value of 37 nM, while positioning the pyrimidine ring next to the benzimidazole group decreased potency more than 6 fold (DB920 had an IC_{50} value of 232 nM). The least potent benzimidazole investigated, DB920, was still over 10 fold more potent than the least potent compound in the benzofuran series.

Fluorescence Properties of Diamidines. The fluorescence spectrum of each diamidine was measured in aqueous solution at a concentration of 500 nM. Fluorescence properties of the diamidines were measured to determine whether the compounds could be investigated with fluorescence microscopy. The UV2A cube set has an excitation bandpass between 330 and 380 nm, and has a long emission bandpass filter, allowing the visualization of emission wavelengths greater than 420 nm. Compound fluorescence in solution is used as an indicator of fluorescence in the trypanosomes. Compounds may have excitation and emission peaks that do not fall in the range of the UV2A cube, and the possibility does exist that the distribution seen in trypanosomes is not the true distribution.

In the benzofuran series, all of the diamidines investigated had similar excitation wavelengths, with most of the compounds having a maximum excitation around 320 nm (Table 5.4). Emission wavelengths varied between 400 and 440 nm, although 4 of the 6 benzofurans investigated had a maximum emission wavelength of approximately 420 nm. The isopropyl substituted 7SAB006 was the most fluorescent analog in this series, and the other isopropyl benzofuran, 1SAB035, had the next highest fluorescence intensity. The two imidazoline derivatives were only weakly fluorescent (Table 5.4).

Compounds in the benzimidazole series had wide ranging fluorescence properties. Five benzimidazoles had maximum excitation wavelengths of approximately 360 nm, but of those five, only two had similar excitation and emission wavelengths (DB915 and DB928, as seen in Table 5.4). Both of these compounds also had furan linker groups. DB818 and DB819 had similar excitation wavelengths, but the imidazoline group in DB819 shifted the emission wavelength by 20 nm to a higher wavelength. DB843 was an interesting compound as it had an excitation wavelength of 360 nm and an emission wavelength of 410 nm (Table

5.4). DB920, the benzimidazole with a pyrimidine linker group had a lower excitation and emission wavelength than the other compounds (λ_{ex}: 320 nm, $\lambda_{em:}$ 390 nm), although there was a small excitation and emission peak at 360 nm and 410 nm respectively. The imidazoline DB819 was the most fluorescent benzimidazole in aqueous solution (Table 5.3).

In vitro Intracellular Distribution in Trypanosomes. The two series of compounds investigated showed different distributions inside trypanosomes. However, the distribution within each series was similar. Of the 6 benzofurans investigated, 5 appeared to be visibly fluorescent inside trypanosomes. Three of the 5 fluorescent benzofurans showed identical distribution patterns, and appeared to localize in a punctate pattern throughout the cell (Figure 5.1). These compounds included the highly potent 150OXD049 and the much less potent 7SAB085 and 7SAB006. 7SAB081 had a similar distribution in the anterior portion of the cells, but also appeared to be localized to the kinetoplast as well. Based on the distribution of the organelles in trypanosomes, it is believed that the organelles are acidocalcisomes, although this has not been confirmed. 1SAB035 appeared to be localized only in the kinetoplast. 6SAB042 was not fluorescent in trypanosomes at the concentration investigated. Additionally, the compound was only weakly fluorescent in an aqueous solution at the same concentration. As most of these compounds have excitation wavelengths that peak around 320 nm, the true distribution of the benzofurans in the trypanosomes may not be seen with the UV2A. In other series of diamidines investigated, fluorescence emission of the compounds in the acidocalcisomes is shifted to a higher wavelength, which could also occur with these compounds, and may explain why these organelles are the only ones visible here.

For the most part, the benzimidazoles were found localized to the nucleus and kinetoplast in trypanosomes (Figure 5.2). Two of the 8 compounds, DB846 and DB920, were not fluorescent in trypanosomes at all. DB846 and DB920 were also 2 of the 3 pyrimidine containing benzimidazoles (Table 5.2). The emission wavelength of DB920 and DB846 was well below the limit of the UV2A cube used for these microscopy experiments (Table 5.3). DB818, DB921, DB819, and DB915 all appeared to be localized to the nucleus and kinetoplast at 8 h. DB843 had the same distribution pattern, but the compound fluoresced yellow inside trypanosomes when viewed under the UV2A cube. Yellow fluorescence with these compounds in the past has been associated primarily with the acidocalcisomes (Chapters 2-4). However, this may be due to the low emission wavelength of this compound, as seen in aqueous solution (Table 5.3). DB928 appeared to be localized to the kinetoplast only (Figure 5.2).

In vivo Accumulation and Distribution of 150OXD049 in Plasma and Trypanosomes.

The accumulation and distribution of 150OXD049 was further investigated in vivo due to its unique intracellular distribution and in vitro antitrypanosomal activity. The compound was curative when administered at a dose of 15 μmol/kg IP (data not shown). When the benzofuran 150OXD049 was administered to infected mice, the compound achieved a peak plasma concentration of 9 ± 5 μM 15 min after administration (Figure 5.3). By 1 h, plasma concentrations had sharply declined to 0.5 ± 0.05 μM. This was followed by a slow decline in concentrations through 24 h, when a concentration of 0.2 ± 0.02 μM 150OXD049 was measured in plasma. Trypanosome concentrations peaked in trypanosomes 1 h after 150OXD049 was administered with a concentration of 111 ± 13 μM inside the cells. The concentration of 150OXD049 in trypanosomes at 24 h was approximately 40 μM

(Figure 5.3). Although lower than previously seen for diamidines such as DB75 and DB820, these concentrations in trypanosomes were sufficient to clear initial parasitemia and cure infected animals over a 60 day period. The $AUC_{0\text{-}24h}$ of 150OXD049 in trypanosomes was 1772 μM*h, while in plasma the AUC was 8.7 μM*h. This represents a 200 fold greater exposure of 150OXD049 in trypanosomes compared to plasma.

In vivo Distribution of 150OXD049 in Trypanosomes. Only 15 minutes after administration of 150OXD049, the compound was found to accumulate within the kinetoplast of trypanosomes (Figure 5.4). By 1 h, the compound has spread to small organelles throughout the cytoplasm, similar in size and location to the acidocalcisomes. After 8 h, it appears that kinetoplast fluorescence has disappeared, although the other organelles were fluorescing brightly. At 24 h, fluorescence was concentrated in the anterior portion of the cell, with numerous organelles showing fluorescence (Figure 5.4). As mentioned previously, it is believed that these organelles are the acidocalcisomes.

E. Discussion

Two series of diamidines, benzofurans and benzimidazoles, were investigated in vitro to determine activity and intracellular localization. This was part of a project aimed at correlating organelle distribution with in vitro activity, and ultimately, evaluating potential mechanisms of action of diamidines against trypanosomes. DNA binding has long been considered a mechanism of action for most diamidines, but the presence of diamidines in other organelles argues that there may different or multiple mechanisms of action. The two series here have strikingly different intracellular distribution, but both series have compounds that are quite potent against trypanosomes.

Although for the most part the benzofurans do not appear to accumulate in the DNA containing organelles of the trypanosome, the nucleus and kinetoplast, they are able to kill trypanosomes in vitro. Previously, one of the primary mechanisms of action of aromatic diamidines has been based on their DNA binding affinity. It appears from the localization of the fluorescent organelles in the cell that the benzofurans accumulate primarily in the acidocalcisomes at 8 h. However, this must be further investigated with probe molecules and antibodies to determine the organelle identity. Additionally, this investigation should be repeated with a fluorescence cube that allows for the visualization of lower excitation and emission wavelengths. Other possible organelles which may accumulate these compounds include the endosomes and glycosomes. Regardless of which organelle is accumulating the benzofurans, this class of compounds is likely to have alternate mechanisms of action that need to be further investigated.

However, due to the fluorescence properties of the benzofuran diamidines (Table 5.4), it is possible that we are unable to see the full distribution of the compounds in

trypanosomes, and this should be considered in future investigations. The maximum fluorescence excitation of most of the benzofurans is lower than the UV2A excitation range, and the emission is at the limit of the fluorescence emission for the UV2A cube set. The impact of DNA binding on fluorescence has been shown to not only enhance or decrease fluorescence, but also to shift fluorescence excitation or emission peaks (data not shown). It is unknown if similar effects occur upon organelle binding, and this should be investigated further.

In the benzimidazole series, all compounds accumulated exclusively in the nucleus and kinetoplast, highlighting the importance of DNA binding to the mechanism of action of these compounds. The least potent compound in the benzimidazole series had an IC_{50} value of approximately 230 nM, compared to the benzofuran series in which the least potent compound had an IC_{50} value greater than 2 μM. Remarkably, unlike the diphenyl furan and aza analogue series (Chapter 4), none of the compounds accumulated in other organelles like the acidocalcisomes. This could potentially highlight a difference in the mechanisms of action between these series of compounds, as the diphenyl furans may have some action in the acidocalcisomes. However, like the benzofurans, it must be cautioned that due to the fluorescence properties of these compounds in solution, the true distribution of these compounds may not be seen in trypanosomes.

It is possible due to the fluorescence properties of both series of compounds that the intracellular distribution seen in trypanosomes is not the true distribution. However, the UV2A cube used for fluorescence microscopy is to our knowledge, the only Nikon cube set available with an excitation band pass filter that extends this low (330 nm). It may be possible in the future to design a fluorescence cube set that allows the visualization of lower

fluorescence excitation and emission wavelengths. Alternatively, another microscope and fluorescence cube set may be utilized. Although comparing the compounds with the UV2A cube set may not allow the visualization of the full distribution of the compounds in trypanosomes, it may still give valid information about the distribution of the compounds in the cells.

In vivo, 150OXD049 was able to cure an acute infection with S427 trypanosomes with trypanosomes concentrations much lower than that previously seen with DB75 and DB820 (Chapter 2). Additionally, when DB820 was administered IP at the same 15 μmol/kg dose, concentrations in trypanosomes were similar to that of the 7.5 μmol/kg IV dose administered in Chapter 2 (data not shown). Although the concentrations of 150OXD049 in trypanosomes were much lower than seen with other compounds, they were sufficient to clear the initial parasitemia, and none of the animals tested relapsed over the 60 day test period used to determine diamidine efficacy. This combined with the unique distribution of 150OXD049 in trypanosomes, is very promising for investigations of the mechanism of action of these compounds.

It has been assumed that selective accumulation of diamidines in trypanosomes has a lot to do with their mechanism of action. If a compound can selectively accumulate within the target cell over other (host) cells, it has a better chance to act on the target cell. With both DB75 and DB820, we have previously seen millimolar concentrations in trypanosomes and 10000 – 20000 fold concentration factors of the diamidines in trypanosomes over plasma. With the benzofuran 150OXD049, this huge concentration in trypanosomes is not necessary for killing trypanosomes, perhaps indicating that different transport mechanisms are used to accumulate the compound in the cells. This needs to be further investigated in the resistant

isolates of the S427 strain which lack the P2 transporter. It is possible that the high concentrations of DB75 and related compounds in trypanosomes is due to multiple targets compounds interact with in the cell, where with 150OXD049, there is only one target within trypanosomes. This unique distribution in trypanosomes has only been seen with the benzofuran diamidines (out of more than 100 diamidines from 8 structural classes investigated). 150OXD049 did appear to initially distribute to the kinetoplast (Figure 5.3), but for the most part was found in the cytoplasmic organelles that we believe are acidocalcisomes.

This diamidine may have a different mechanism of action against trypanosomes than DB75, which opens many new avenues for potential study of new diamidines. With this series, the benzimidazoles, and the diphenyl furans, three series of compounds exist that may have different mechanisms of action. While the benzimidazoles appear to be found only in the DNA containing organelles, select compounds in the diphenyl furan series, like DB75, also accumulate in the acidocalcisomes. Finally, the benzofurans appear to accumulate almost exclusively in the organelles believed to be acidocalcisomes. These compounds especially will be useful for studies investigating what diamidines do in the acidocalcisomes. While in the diphenyl furan series, it can be argued that there is little reason other than physicochemical properties for diamidine accumulation in acidocalcisomes (i.e. their high pKa values leading to sequestration in organelles with low pH); compounds in the benzofurans must be exerting some mechanism of action in this organelle in order to kill trypanosomes. Acidocalcisomes have been proposed to be involved in calcium and pH homeostasis in trypanosomes (2), and benzofurans may interfere with those processes. There

are also enzymes found in the acidocalcisomes that could be inhibited by benzofuran diamidines.

It would also be interesting to investigate the possibility of synergism between compounds in the benzofuran and benzimidazole series, since these compounds likely have different mechanisms of action. In the future, there may be a need for co-administration of two diamidines with differing mechanisms of action in an attempt to circumvent drug resistance.

The two series of compounds investigated were selected from many other series investigated based on their differing distribution properties. While the benzimidazoles represent the typical distribution pattern expected of diamidines that potently bind DNA, the benzofurans seem to have a unique and different distribution pattern for the most part. The benzofurans will be exciting compounds for future study. Not only is there the potential for different accumulation mechanisms to bring the compounds inside trypanosomes, they also likely have a novel mechanism of action. The compounds will be useful for mechanism of action studies, but also could represent a new approach for the future development of antitrypanosomal diamidines, targeting other organelles besides the DNA containing nucleus and kinetoplast.

Table 5.1: Summary of diamidine compounds investigated					
	Total Number	Fluorescent Compounds[2]	Non - Fluorescent Compounds[2]	IC_{50} < 10 nM	10 nM<IC_{50} < 100 nM
Diphenyl furans	24	18	5[*]	6	9
Benzofurans	9	5	4	2	4
Benzimidazoles	15	14	1	8	4
Bis-benzimidazoles	5	5	0	1	3
Fused Rings	9	5	4	3	2
Terphenyls	11	4	6	2	9
Alkyl linkers[#]	11	1	10	4	2
Isoxazoles[1]	7	0	7	2	3
Total	91	52	37	28	36

[*]One compound too toxic for testing, [#]Differ in structure, but all generally have alkyl linkers, [1]also oxadiazoles and related compounds, [2]indicates fluorescence of the compounds seen using UV2A Microphot microscope. All of the compounds were fluorescent to some extent.

Table 5.2: Structure and In Vitro Antitrypanosomal Activity of Benzofuran Diamidines

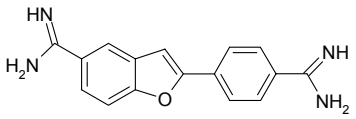
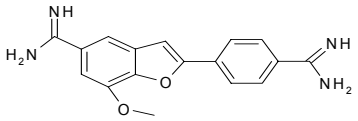
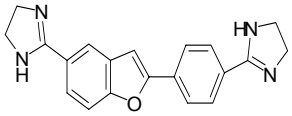
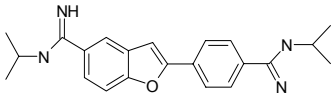
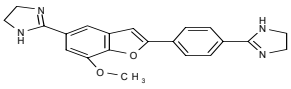
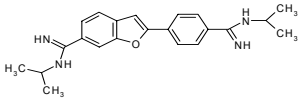
Compound	Structure	IC_{50} (nM)
150OXD049		11
7SAB081		12
6SAB042		59
1SAB035		510
7SAB085		2343
7SAB006		3151

Table 5.3: Structure and In vitro Antitrypanosomal Activity of Benzimidazole Compounds

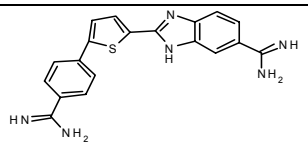
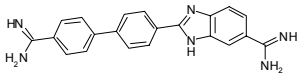
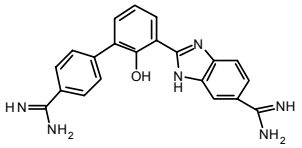
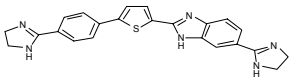
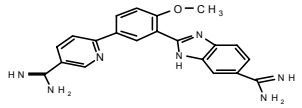
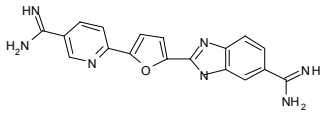
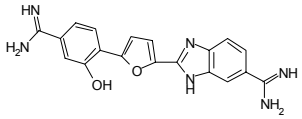
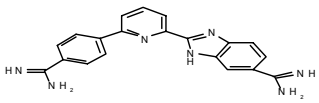
Compound	Structure	IC_{50} (nM)
DB818		0.9
DB921		1.2
DB843		1.9
DB819		3.6
DB846		6.7
DB915		37
DB928		90
DB920		232

Table 5.4: Fluorescence Properties of a 500 nM aqueous solution of Benzofuran and Benzimidazole Diamidines

Compound	Maximum Excitation Wavelength (nm)	Maximum Emission Wavelength (nm)	Fluorescence Intensity
150OXD049	325	426	444
7SAB081	320	420	5.1
6SAB042	310	420	3.3
1SAB035	320	420	514.7
7SAB085	323	440	17.2
7SAB006	326	400	936.5
DB818	360	430	24.4
DB921	320	434	52.8
DB843	359	410	2.5
DB819	360	450	487
DB846	322	390	21.7
DB915	358	470	215.7
DB928	360	467	145.6
DB920	320	390	240

Figure 5.1: In vitro distribution of benzofuran diamidines in trypanosomes. On left is phase contrast image, on right is fluorescence image.

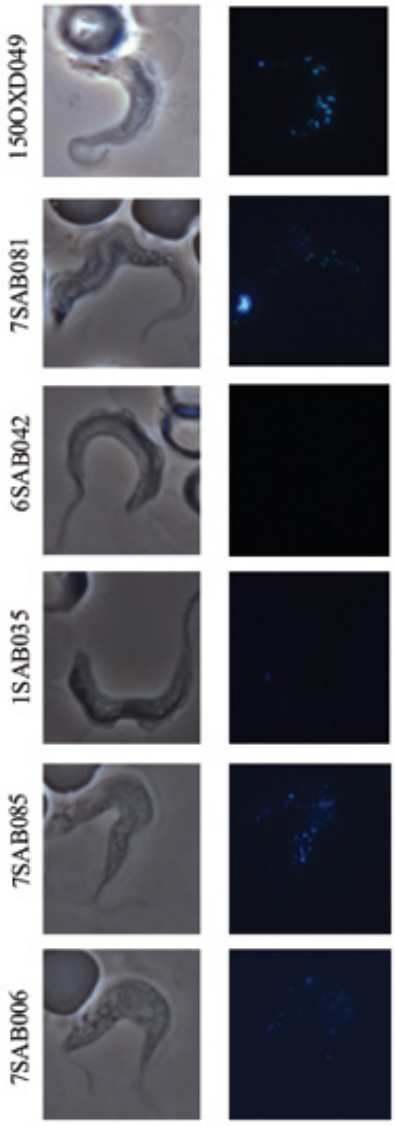


Figure 5.2: In vitro distribution of benzimidazole diamidines in trypanosomes. On left is phase contrast image of trypanosomes, and on the right is the corresponding fluorescence image.

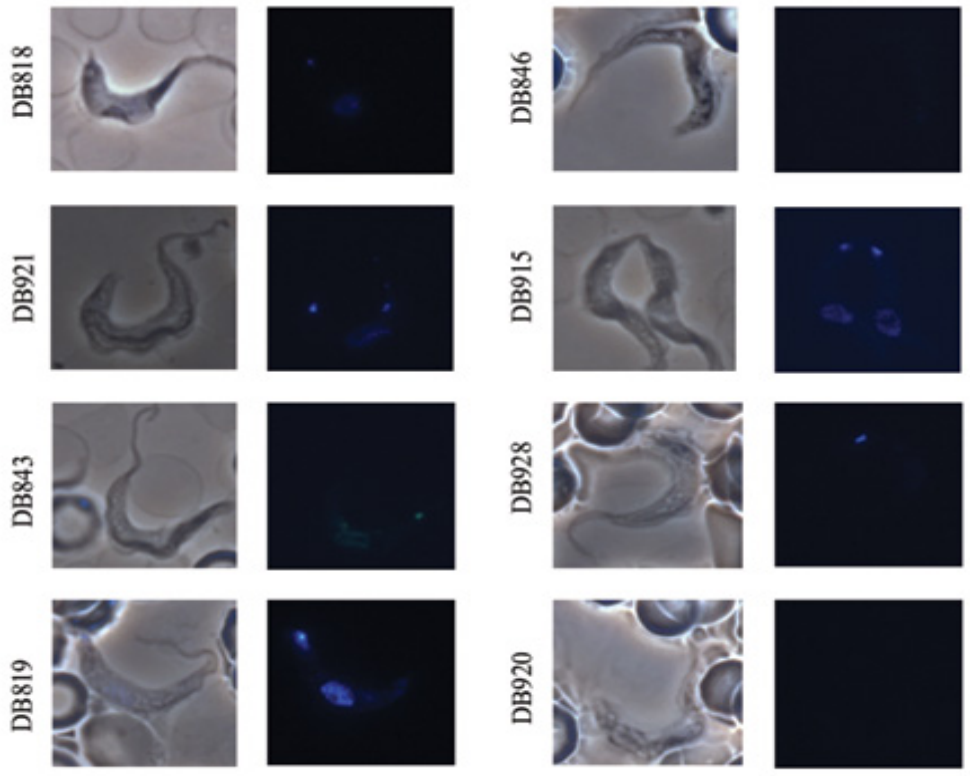


Figure 5.3. In vivo Accumulation of 150OXD049 in Trypanosomes and Plasma. Concentrations are presented as μM ± SE (n=3-5 mice).

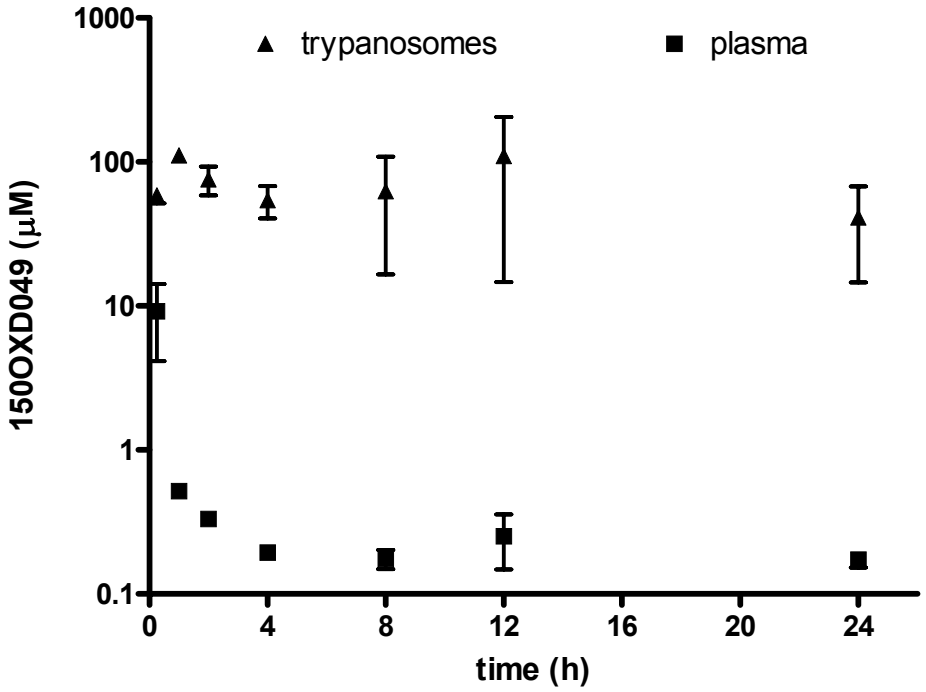
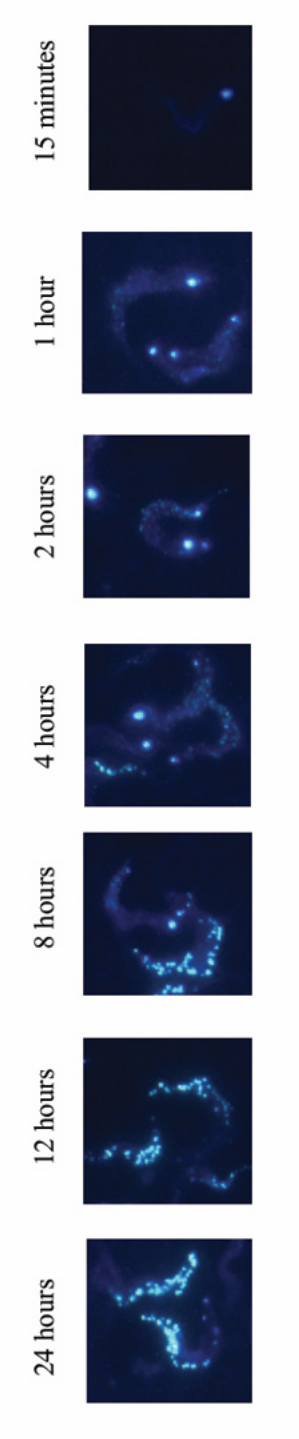


Figure 5.4. In vivo Distribution of 150OXD049 in trypanosomes.



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CHAPTER 6

CONCLUSIONS

A. Conclusions

Aromatic diamidines have long been used for the treatment of parasitic diseases such as human African trypanosomiasis. The investigations described here have focused primarily on diphenyl furan and aza analogues related to DB75 and DB820 (Chapters 2-4). Chapter 5 introduced two additional series of compounds which may be useful for future investigations of mechanism of action or drug development. Before these studies began, little was known about the trypanosomal distribution and accumulation of diamidines. Metabolism, transport and pharmacokinetics of the prodrug DB289, or pafuramidine, had been investigated for several years in several models (22, 27, 29, 32, 33). The investigations presented here have added essential information about the disposition of diamidines in their target organism.

The diphenyl furan diamidine DB75, and its aza analog DB820, are both very potent diamidines against trypanosomes. Both compounds have IC_{50} values of less than 10 nM, and have potent in vivo efficacy against several mouse models of trypanosomiasis (Chapter 2). Investigations showed that both DB75 and DB820 accumulated to high concentrations in trypanosomes, 10000-20000 times plasma concentrations. This is indicative of selective transport into trypanosomes, likely through uptake transporters such as the P2 transporter. As hypothesized, the strong DNA binding DB75 and DB820 were found to accumulate in the two DNA containing organelles of trypanosomes, the nucleus and kinetoplast, and likely lead to killing of trypanosomes by accumulating there. However, acidocalcisomes were also found to accumulate DB75 and DB820. This could be due to the high pKa values of these diamidines (pKa's are approximately 12) causing sequestration in these acidic organelles by the pH-partitioning theory, or the diamidines may exert some mechanism of action in these organelles.

That diamidines accumulated to high concentrations in trypanosomes was not unexpected. Previously, pentamidine was found to accumulate to concentrations of approximately 0.8 mM in trypanosomes isolated from infected rats 4 h after administration (4). However the distribution of pentamidine cannot be easily investigated in trypanosomes as the compound is not fluorescent. Investigations of the intracellular distribution of DB75 and DB820 are the first to give confirmation that the compounds actually accumulate at the hypothesized targets.

Transport experiments for DB75 have indicated that the compound accumulated rapidly in trypanosomes primarily by only one transporter, the P2 transporter (13). Transporter based drug resistance has been shown to be a problem for compounds such as melarsoprol in clinical settings (17), and this problem must be considered for DB75 as well. Three trypanosome strains have been created in a laboratory setting which lack uptake transporter activity. *TbAT1-/-* is a trypanosome line in which the alleles for the P2 transporter (TbAT1 gene) have been removed (18). DB75R-CL1 is a clone made resistant to DB75 in vitro (13), while B48 is the *TbAT1-/-* made resistant to pentamidine (3). B48 has also lost functional activity of the HAPT1 transporter. These three trypanosome lines allowed us to determine long term accumulation of DB75 in trypanosomes for the first time in the absence of the primary uptake transporter. Surprisingly, DB75 still accumulated to high concentrations in vitro, albeit to lower concentrations than seen in the wild type S427 line despite all these strains being resistant to DB75 in vitro. The high concentrations found in the resistant strains in vitro has raised several questions about the environment (i.e. the media) that experiments are conducted in. CBMEM is a highly supplemented medium, which in addition to many amino acids, also includes high concentrations of adenosine,

hypoxanthine, and inosine. High concentrations of these purines may have some effect on the presence, activity, and regulation of transporters expressed on the surface of trypanosomes.

The concentration of adenosine in CBMEM media is approximately 80 μM. Adenosine competitively inhibits uptake of DB75 in trypanosomes with an IC_{50} value of 0.8μM, after 5 min incubation with 1 μM radiolabeled DB75 and various concentrations of adenosine (12). The 100 fold excess of adenosine in the medium may have saturated the P2 transporter for DB75 transport. It is possible that the accumulation seen in trypanosomes, both drug sensitive and resistant, is due to another transporter that is active when the P2 transporter is saturated. Concentrations of adenosine in human blood have been reported to be in the range of 0.1- 1 μM. The half life of adenosine in the blood is very short (<1 s) (20), which keeps concentrations low.

In vivo, however, accumulation in the resistant strains was greatly reduced in comparison to the wild type line, especially when administered at a low dose (5 μmol/kg). Concentrations in the resistant trypanosomes were less than 20% of concentrations in the wild type S427 trypanosomes. This dose was not able to cure any of the resistant lines tested, likely due to the low concentrations that accumulate in these trypanosomes. Even at the higher dose investigated the resistant strains accumulated much less DB75 than the wild type line.

In vitro and in vivo distribution of DB75 in the resistant lines was similar. Consistent with the reduced accumulation, distribution of DB75 in trypanosomes progressed slower than that seen in the wild type line. DB75 first appeared in the kinetoplast, then nucleus, and much later, in the acidocalcisomes. This delayed distribution was also seen when DB75 is

incubated with wild type S427 trypanosomes at 4 ºC (preliminary studies described in Appendix II). It is possible that the same mechanism leads to accumulation of DB75 in trypanosomes at 4 ºC and in the absence of the P2 transporter, though this needs further investigation.

Investigation of diphenyl furans related to DB75 and analogues of DB820 yielding interesting results. It was hypothesized that accumulation in trypanosomes would be related to in vitro activity, and possibly even in vitro distribution. In other words, it was expected that more potent diamidines would accumulate in trypanosomes to higher concentrations than less potent diamidines. However, this was not the case in the diphenyl furan series, as two compounds of intermediate potency accumulated to higher concentrations than the very potent DB75. DB690, a 2,4 diphenyl furan analogue, which was more than 3 fold more potent than DB75, did accumulate to peak concentrations 6 fold higher than that of DB75. All of the compounds in the diphenyl furan series were potent DNA binders, regardless of substitutions on the diamidine compounds. Additionally, all of the diphenyl furans were found to be localized to the nucleus and/or kinetoplast of trypanosomes, supporting DNA binding as a major mechanism of action of the compounds.

Compounds in the DB820 series yielded similar results. Again there was no correlation between accumulation in trypanosomes over time and activity. The most potent compound in the series did accumulate to the highest concentration in trypanosomes, but every compound tested accumulated to higher concentrations than DB820, the second most potent compound in the series. There may be a minimum accumulation necessary for activity of the diphenyl furans and aza analogues against trypanosomes – approximately 1 mM.

In an effort to expand investigation of diamidines to other series of compounds, more than 100 compounds from 8 different classes were examined. In vitro activity and distribution experiments with benzofuran diamidines and benzimidazole diamidines showed different distribution between the two series. While the benzimidazoles distributed to the nucleus and kinetoplast, several compounds in the benzofuran class were localized to organelles in the trypanosomes believed to be the acidocalcisomes based on their localization in trypanosomes. Of the compounds investigated, only 2 appear to be localized to the kinetoplast, 1SAB035, and 7SAB081, which is also located in the cytoplasmic organelles. Accumulation in these organelles may represent a novel mechanism of action for the benzofurans, which must be further investigated.

One of the benzofurans, 150OXD049, was investigated in vivo to determine accumulation in trypanosomes and plasma. Unlike DB75 and DB820, this benzofuran was not highly concentrated in trypanosomes over plasma; there was only a 200 fold concentration in the parasite over plasma. Despite this, the compound was curative in infected animals. The compound may accumulate in trypanosomes by different mechanisms than DB75 and DB820, leading to less compound overall in trypanosomes. The related compound, DAPI, has affinity for all three uptake transporters found on trypanosomes, but transport is not mediated by the P2 transporter primarily (3). In the B48 line, 1 mM pentamidine has been shown to prevent the fluorescence of DAPI in trypanosomes over 60 min. With the structural similarities between DAPI and the benzofurans, it is possible that the compounds have similar uptake properties. To my knowledge, long term accumulation of DAPI in trypanosomes has not been investigated. Additionally, the lower accumulation in trypanosomes may be related to the mechanism of action of 150OXD049. Since there is

predominantly only one target in the cells that these compounds accumulate in, it may not be necessary to accumulate millimolar concentrations of 150OXD049 in trypanosomes. DB75 and DB820 potentially have at least three targets in trypanosomes – the nucleus, kinetoplast and acidocalcisomes.

The results with 150OXD049 indicate that the hypothesis of DNA binding being essential to the mechanism of action of diamidines may not be true for all diamidine compounds. It is likely that such a generalization can not be made about all diamidine compounds. Investigation of only 100 diamidines has potentially turned up two different mechanisms of action. Future experiments should be planned to identify the mechanism of action of the benzofurans. Also it may be prudent to include other targets for future diamidine compounds to be developed.

These investigations have yielded promising results, and raised questions for future studies. Diamidines accumulate to high concentrations in trypanosomes, and localize to several intracellular targets. The most intriguing results were found with the trypanosome lines lacking uptake transporters. DB75 concentrations in these lines, although reduced, were still high. These results require further study to determine whether drug resistance to DB75 will be a problem with clinical use. Additionally, investigations on the mechanism of action of diamidines can be started with information already available about the distribution and accumulation of diamidines in trypanosomes.

B. Future Directions

Much remains to be investigated with aromatic diamidines. Pafuramidine, the prodrug of DB75, may be on the market in a few years as a treatment for trypanosomiasis, and the mechanism of action of this compound is still unknown. Knowing the accumulation and distribution of these compounds is the first step to determining mechanism of action of these diamidines. Additionally, the complete mechanisms for accumulation of diamidines like DB75 need to be determined.

Fluorescent compounds like the diamidines investigated here will useful for further investigation of mechanism of action. Qualitatively, the distribution of the compounds is known using fluorescence microscopy. Isolation of organelles will allow the study of accumulation in the various target organelles, and will allow the investigation of potential targets in the isolated organelles. Separation of the two DNA targets will enable the study of affinity for both the kDNA, which is more AT rich, and the nuclear DNA as well inhibition of the various DNA associated enzymes like topoisomerase in the both organelles. It is possible that a diamidine could have greater affinity to inhibit the action of topoisomerase in the kinetoplast than the nucleus, or vice versa. A preliminary investigation on the isolation of organelles is presented in Appendix III. With the isolation studies, at least one organelle has bee isolated definitively, although purity is unknown.

The use of inhibitors which block accumulation in certain organelles is another way to investigate mechanism of action. Altering the acidity of acidocalcisomes with the sodium ionophore monensin has already been used to prevent accumulation of DB75 in acidocalcisomes (Appendix I). These studies should be expanded to other inhibitors to verify whether DB75 and other compounds exert any action in the acidocalcisomes. The ability of

diamidines to inhibit phosphatases and other enzymes found in the acidocalcisomes should be investigated as well. Several acidocalcisome deficient trypanosome lines have also been developed. These lines have been created though genetic modification of genes found expressed in the trypanosome acidocalcisomes (14, 15). These trypanosomes could be used to investigate mechanisms of action of diamidines in the acidocalcisomes.

Another way to study mechanism of action would be to use electron microscopy. In the past, transmission electron microscopy has been used to examine effects of antitrypanosomal compounds on trypanosome ultrastructure. For example, pentamidine has been shown to have an effect on kinetoplast structure using this technique (31). TEM investigations will be useful to investigate whether any differences exist between the wild type and drug resistant strains of trypanosomes (i.e. are the compounds not only accumulating in the trypanosomes to a lesser extent, but also accumulating less in the targets of interest?).

The question of trypanosome drug resistance in the field is a major one that needs to be investigated, not only for aromatic diamidines, but also for all compounds currently used. For the diamidines, it is vital that the mechanisms of accumulation by all potential transporter or transport processes are determined. For administration of DB75 itself in vivo, it appears from the experiments already conducted that the P2 transporter has an effect on accumulation of DB75 over time. However, the accumulation of high micromolar concentrations when the primary transporter to accumulate the drug is missing is intriguing. Based on this work, it is clear that DB75 accumulates in trypanosomes via some other means, and this mechanism remains to be elucidated. Previous work has determined that two P1 – type transporters are

upregulated in *TbAT1-/-* trypanosomes, but to date, these transporters have not been shown to be involved in drug uptake (10).

In vitro experiments investigating accumulation of DB75 in these resistant lines need to be expanded to other compounds in the available library, including DB820 and DB829, two lead diamidines for treatment of late stage trypanosomiasis. Furthermore, the experiments should be expanded to investigate the accumulation in protein free buffer systems, as well as adenosine free media. The growth media used for the experiments described in this dissertation was also used for accumulation experiments. This media contains relatively high concentrations of adenosine and other purines. Although these concentrations should not saturate the P2 transporter, it should be investigated by eliminating first the adenosine in the media, and then other media components. The data presented here indicated that over time a higher concentration of DB75 accumulated in drug resistant trypanosomes than would be expected based on short term experiments conducted on trypanosomes ex vivo in a simple buffer system (13). It is also unknown whether absence of the P2 transporter and HAPT1 activity has led to the upregulation of another transport mechanism in the drug resistant trypanosomes.

Experiments must also be conducted to compare accumulation of pentamidine, propamidine, and berenil in wild type and drug resistant trypanosomes. Pentamidine and propamidine are both substrates for the HAPT1 transporter, while berenil, like DB75, is only thought to be a substrate for the P2 transporter. These three compounds have been widely studied, and much is known about their uptake properties. Differences in the uptake of these compounds will be investigated by comparing their long term accumulation to that of DB75. Additionally, accumulation of phenyl arsine oxide, a lipophilic derivative of melarsoprol

should be investigated. Studies have shown that the in vitro activity of this compound is unchanged in the resistant lines investigated here. As the compound is lipophilic, it should passively diffuse across trypanosome membranes and can be used as a control for accumulation in various media and buffer systems.

The discrepancy in accumulation of DB75 in the drug resistant strains at the lowest dose administered (5 μmol/kg IP) led to the idea that accumulation of DB75 in trypanosomes should be determined after curative doses of DB289 are administrated. After administration of DB75, concentrations of the compound in plasma are initially high, but decline rapidly over time. After administration of DB289, concentrations of DB75 in plasma will rise slower, and may be elevated longer. Regardless, accumulation of DB75 in trypanosomes after administration of DB289 is more clinically relevant than administration of DB75 alone. The possibility that higher doses or longer dosing administrations can overcome the potential for drug resistance in these strains may also be investigated.

The resistant lines studied so far were all developed in the laboratory. Clinical isolates resistant to melarsoprol have been found to have mutations in the P2 transporter (17), but the lines used in this study had the transporter completely removed. It is possible that even with several mutations to the transporter as seen in the clinical strains, some level of accumulation in trypanosomes may still occur using this transporter. It may be prudent to investigate accumulation of DB75 and related compounds in these clinical strains to determine whether the laboratory created strains are a relevant model.

Investigations of other aromatic diamidines should continue for development of compounds not only for trypanosomiasis but for other parasitic diseases as well. Already compounds with different distribution and likely different mechanisms of action in

trypanosomes have been identified, after investigating only 100 diamidines. Although it is unlikely that any more classes of compounds with different distributions will be discovered, these investigations have set the groundwork for the further study of diamidines in trypanosomes.

These proposed future studies will add extensively to our knowledge of diamidine accumulation, distribution, and mechanism of action in trypanosomes. Much remains to be learned about DB75 and other diamidines. Studies proposed here will not only add to the knowledge of diamidines, but may also aid in future drug development efforts not only for trypanosomes, but also for other parasitic diseases.

APPENDIX 1

ACCUMULATION IN THE ACIDOCALCISOMES IS NOT ESSENTIAL FOR THE MECHANISM OF ACTION OF DB75

Introduction

Exact mechanisms of action of diamidines such as DB75, despite intensive study, are still poorly understood. DB75 and related diamidines are known to bind DNA tightly in AT rich regions (2). There are many hypotheses for the mechanism of action of diamidines in addition to DNA binding, including interference with DNA associated enzymes such as topoisomerases, interference with polyamine activity, and protease inhibition (30). Pentamidine, an early analog of DB75, has been shown to linearize kinetoplast DNA and interfere with topoisomerase activity in the kinetoplast (25, 26). While distribution of nonfluorescent pentamidine cannot be followed in trypanosomes, fluorescent DB75 has been shown to accumulate in the kinetoplast and nucleus where it binds to DNA. Further experiments are needed to determine whether DB75 affects topoisomerases found in both organelles or linearizes kinetoplast DNA. However, DB75 is also thought to be localized to the acidocalcisomes, recently discovered organelles in parasites such as trypanosomes.

The acidocalcisomes, as their name suggests, are acidic organelles that sequester calcium ions, as well as other ions, and polyphosphates (7). The organelles represent up to 2% of the total trypanosome volume, and their size is inversely proportional to their number. In other words, the more acidocalcisomes present in the cell, the smaller the organelle volume. Several transporters are known to be localized to the surface of acidocalcisomes, including a Ca^{2+}-ATPase that is sensitive to vanadate, and a vacuolar H^{+}-ATPase (7). Also found on the membrane of acidocalcisomes are various ion exchangers, such as Na^{+}/H^{+} and Ca^{+}/H^{+} exchangers, responsible for maintaining the ionic balance in the organelle and trypanosomes (7). Alkalinizing agents such as the ionophores monensin and nigericin, and ammonium chloride (NH_4Cl), have also been shown to release Ca^{2+} and acridine orange from

acidocalcisomes (7, 8). Acidocalcisomes are thought to be involved in many intracellular processes such as osmoregulation, pH regulation and the storage of ions (7, 8). However, any role that DB75 may play in these organelles is unknown. This study was initiated to investigate whether accumulation in the acidocalcisomes is essential to the accumulation of DB75 in trypanosomes.

Methods

Trypanosoma brucei brucei S427 trypanosomes were cultured in CBMEM media as previously described (16). For various time points from 5 min - 1 h, 10^6 trypanosomes were pretreated with 5 μM monensin (stock solution prepared in ethanol) or 20 mM ammonium chloride. After pretreatment, trypanosomes were washed and then DB75 was added to a final concentration of 7.5 μM. DB75 was incubated with trypanosomes for 2 h, and then trypanosomes were washed twice and resuspended in freshly isolated mouse blood. Thin films were prepared from a drop of blood, and viewed with a Nikon Microphot FXA microscope equipped with a 60 DM x 1.4 NA objective lens, a mercury lamp, and QImaging Micropublisher 3.3 CCD Digital Camera (QImaging Corporation, Surrey, BC, CA) was used for fluorescence microscopy. QCapture version 3.0 imaging software was used to capture images (QImaging). The microscope was equipped with a Nikon UV2A cube that limits excitation wavelengths to 330-370 nm and emission wavelengths to ≥ 420 nm. Additionally, DB75 and monensin or NH_4Cl were co-incubated with trypanosomes for the 2-4 h time period as well. Acridine orange (Sigma) was used as a marker for acidocalcisome accumulation. Acridine orange has been shown in the past to accumulate in acidocalcisomes and be displaced by alkalinizing agents (8). Concentrations of DB75 in trypanosomes with

and without a monensin pretreatment were determined by High Performance Liquid Chromatography (HPLC) as previously described (16).

Results

After two hour incubation in vitro with trypanosomes, DB75 accumulated in the nucleus, kinetoplast and acidocalcisomes (Figure 1, first column). However, when ammonium chloride or monensin were used as a pretreatment before DB75 was added, accumulation in the acidocalcisomes was not seen (Figure 1, columns 2 and 3). A 60 min pretreatment of ammonium chloride was needed to prevent DB75 accumulation in acidocalcisomes, although partial inhibition of acidocalcisomes localization was seen after 30 min. The sodium ionophore monensin, on the other hand, only needed to be incubated with trypanosomes for 15 min before DB75 was added to abolish acidocalcisome accumulation. Partial inhibition of accumulation was seen after 5 min. Monensin completely abolished acridine orange fluorescence in the acidocalcisomes of trypanosomes after only 15 min (data not shown).

Interestingly, when accumulation of DB75 was determined by HPLC analysis, there was no significant difference in trypanosome concentrations with or without the monensin pretreatment (Figure 2) after either 1 or 2 h. This indicates that when DB75 is unable to accumulate in acidocalcisomes, it must redistribute to other organelles, and also that monensin pretreatment does not inhibit overall accumulation of DB75 in trypanosomes.

Conclusions

Monensin and ammonium chloride inhibited the accumulation of DB75 in acidocalcisomes, similar to effects seen previously with monensin and acridine orange (8). Treatment with both monensin and ammonium chloride serve to increase alkalinity in acidic

organelles like acidocalcisomes. Therefore, inhibition of acidocalcisome accumulation could be related to increased pH in the acidocalcisomes caused by either treatment. Additionally, monensin has been shown to cause the release of calcium ions from acidocalcisomes into the cytoplasm of trypanosomes (28). It is unknown by what mechanism DB75 accumulates in the acidocalcisomes (i.e. by what transporter if any), but this should be further investigated with inhibitors of the various uptake transporters on the surface of trypanosomes (for review see (7)).

Despite the lack of accumulation of DB75 in acidocalcisomes after pretreatment of monensin (30 min), overall concentrations in the cells were unaffected. This could be due to redistribution of available DB75 to other organelles, such as the nucleus and kinetoplast (Figure 1). The next logical step would be to determine whether monensin affects the IC_{50} value of DB75. In preliminary studies, we have found that monensin is toxic to the cells over long periods of time (72 hours for the in vitro activity experiment). It is not known how long the effects of monensin last in the cells. We plan to further investigate other potential inhibiting agents to determine their effects on DB75 accumulation, distribution and activity. Then the role that the acidocalcisomes play in the mechanism of action of DB75 can be fully assessed.

Figure 1: Distribution of DB75 (7.5 μM) in trypanosomes alone (first column A and B), and after either a 60 min pretreatment of ammonium chloride (NH_4Cl) or a 15 min pretreatment of monensin.

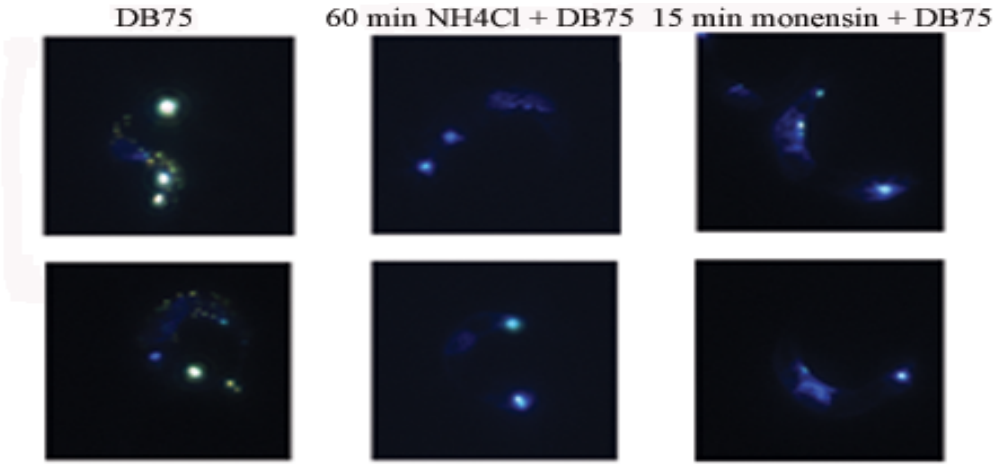
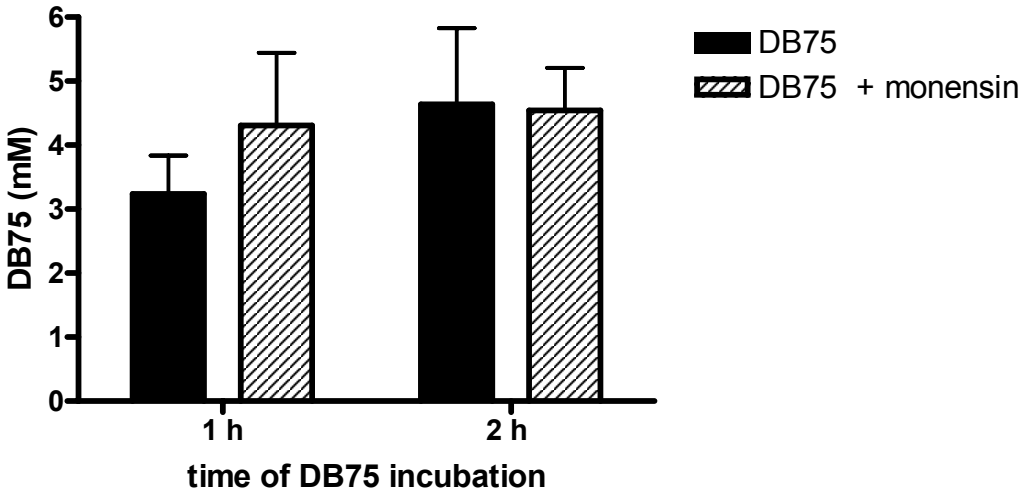


Figure 2: Accumulation of DB75 in trypanosomes alone and after a pretreatment of monensin (5 μM). Student t-tests showed no difference in accumulation of DB75 in trypanosomes with or without monensin pretreatment.



APPENDIX II

TEMPERATURE EFFECTS ON ACCUMULATION AND DISTRIBUTION OF DB75 AND DB249 IN AFRICAN TRYPANOSOMES

Introduction

DB75 and DB249 have been shown to accumulate to high concentrations in trypanosomes in vitro (CHAPTER 4). Accumulation of diamidines in trypanosomes is thought to occur mainly through uptake transporters on the surface of the cell. These transporters include the P2 adenosine transporter, and the HAPT1 and LAPT1, which are known to accumulate pentamidine specifically in trypanosome (5, 6). Previous work has shown that transport of DB75 is reduced when the P2 transporter is missing (CHAPTER 3), but accumulation still occurs in trypanosomes.

Following work done in CHAPTER 4, showing very high accumulation of several diamidines over an 8 h period, additional experiments were planned to investigate mechanisms of long term accumulation of diamidines in trypanosomes. Here data is presented showing accumulation of two diamidines, DB75 and the cyclohexyl derivative DB249, at 4 ºC. Short term accumulation experiments have shown minimal accumulation of DB75 in trypanosomes at low temperatures (13).

Methods

Trypanosomes were cultured as previously described (16). DB75 and DB249 (7.5 μM) were incubated with approximately 10^6 trypanosomes/ml for time periods between 1 and 4 h at either 4 ºC or 37 ºC. At time points after administration of compound, cells were washed twice with drug free media and resuspended in fresh media. The compounds were extracted and analyzed as described in Chapter 4. Additionally, DB75 and DB249 were incubated with 7.5 μM to determine intracellular distribution in trypanosomes at both temperatures by microscopy. After 1 h, cells were washed twice and resuspended in freshly

isolated mouse blood. Thin smears were prepared and viewed using a Nikon Microphot FXA microscope equipped with a UV-2A cube.

Results

Accumulation of DB75 and DB249 (a cyclohexyl substituted derivative of DB75) was reduced at 4 ºC. The accumulation of DB75 at 4 ºC was only about 40% of the accumulation seen at 37 ºC. DB249 accumulation at 4 ºC, on the other hand was 75-90% of accumulation at 37 ºC. This decrease in accumulation at 4 ºC was not significant ($p = 0.3$, 0.4 for 1 h and 4 accumulation of DB249). P values associated with the decrease in DB75 accumulation with decreasing temperature were 0.1 and 0.03 for 1 h and 4 h accumulation experiments respectively).

Distribution studies showed that both DB75 and DB249 accumulate within the trypanosomes at 4 ºC, although distribution is not as extensive as it is at the higher temperature (Figure 2). After 1 hour at 4 ºC, DB75 is localized to the nucleus and kinetoplast of trypanosomes, while DB249 is found only in the kinetoplast. After a 37 ºC incubation for 1 h, DB75 is localized to the kinetoplast and nucleus, and the kinetoplast fluoresces white, typically associated with high concentrations of DB75 in the organelle. At 37 ºC, DB249 is found in the nucleus and kinetoplast and appears to be much brighter in these organelles than at 4 ºC.

Conclusions

The results shown here indicate that both DB75 and the more lipophilic DB249 accumulate in trypanosomes when cells are maintained at 4 ºC. For the 4 ºC experiments, cells were chilled before the experiments began to ensure that trypanosomes had reached that temperature before the experiments began. It has been postulated that perhaps the positively

charged compounds were only associated with the surface of trypanosomes at low temperatures and were not accumulating in the cells, but here we show that both compounds are actually localizing in the cells to the theoretical target organelles. However, the mechanism for this accumulation is still unknown. Using low temperatures, we were attempting to stop any active transport processes from occurring, but trypanosomes can retain motility and viability at 4 ºC for long periods of time, assuming they are provided with basic nutrients like glucose (data not shown). Since motility of trypanosomes requires energy, it is likely that we have not slowed or stopped energy dependent processes over the period of time studied. Therefore, the mechanism of this uptake at 4 ºC can not exclude the possibility of a transporter such as P2 operating at this temperature. However, other investigators have used transport at 4 ºC to indicate passive diffusion, albeit over shorter periods of time (1). Interestingly, the pattern of accumulation in the organelles of trypanosomes is similar to that seen with the P2 transporter knockout trypanosomes, with accumulation first occurring in the kinetoplast, then nucleus (seen clearly with DB249). The possibility exists that whatever mechanism leads to the accumulation of DB75 in *TbAT1-/-,* DB75^{R}-CL1, and B48 lines, is responsible for the accumulation of these compounds at 4 ºC.

Additional experiments were employed using adenosine (1 mM, in addition to the adenosine found in media) to block accumulation of DB75, but concentrations of DB75 were not substantially reduced. It remains to be investigated whether accumulation of DB75 in adenosine free media is lower or higher than in the current growth media. Perhaps the adenosine in the media currently used has saturated the P2 transporter, so that any accumulation of DB75 seen is through an alternative route. There is still much to be investigated in terms of diamidine accumulation in trypanosomes.

Figure 1: Relative Accumulation of DB249 and DB75 at 37 ºC and 4 ºC. Accumulation at 37 ºC at 1 hand 4 h for both compounds is indicated by closed bars, while accumulation at 4 ºC at 1 h and 4 h for both compounds is indicated by open crosshatched bars.

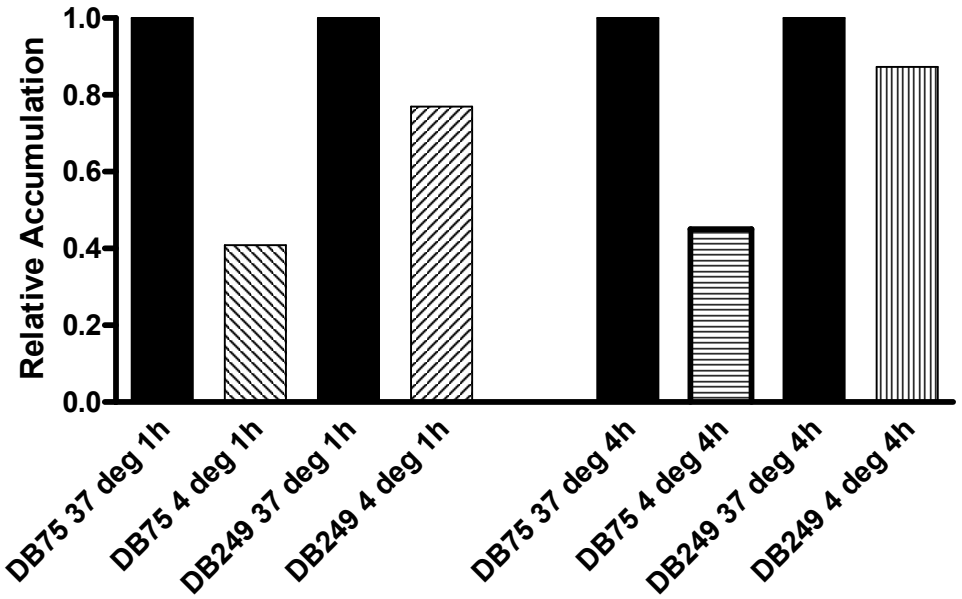
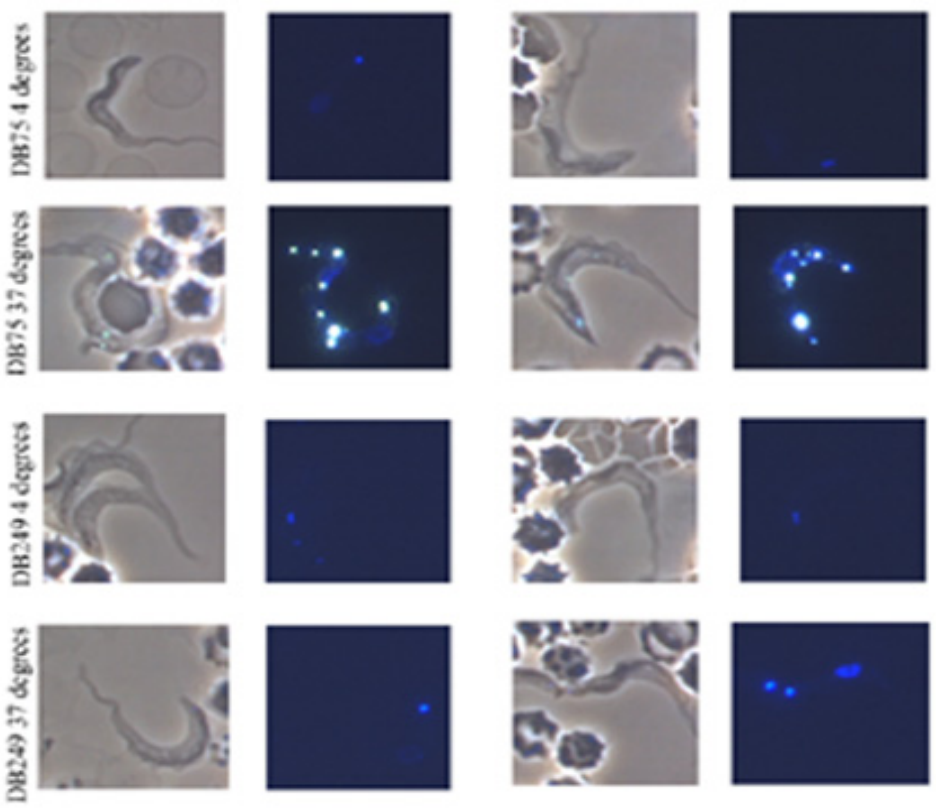


Figure 2: Distribution of DB75 and DB249 in trypanosomes at 4 ºC and 37 ºC. Phase contrast images are included to visualize trypanosome orientation. Two images at each temperature are provided.



APPENDIX III

ISOLATION OF TRYPANOSOME ORGANELLES: PRELIMINARY STUDIES FOR INVESTIGATING MECHANISM OF ACTION

Introduction

There are several methods in which mechanism of action of diamidines can be determined. An organelle isolation method was chosen. This method has been utilized in drug sensitive and drug resistant cancer cells to determine that localization of doxorubicin or daunorubicin was altered in drug resistant cells (9);(Mathis, unpublished results). An attempt to isolate organelles from trypanosomes in which diamidines like DB75 accumulate was made. DB75 has been shown to accumulate in the kinetoplast of the mitochondrion, the nucleus and acidocalcisomes. After isolating organelles, the amount of compound in each organelle could be determined by HPLC or LC/MS analysis. A mass balance approach could be used to determine the relative contribution that each organelle makes to overall trypanosome concentrations.

Since qualitatively, the distribution of DB75 in trypanosomes was known, isolation procedures for several organelles, including the nucleus, kinetoplast and mitochondria, acidocalcisomes, lysosomes, and glycosomes were investigated. This was the first step to future investigation of DB75 accumulation in organelles. Preliminary data here shows the isolation of acidocalcisome fractions.

Methods and Materials

Trypanosome Isolation. Trypanosomes were isolated from rats infected with 10^6 trypanosomes 4-5 days post infection. All animal experiments adhered to guidelines outlined by the University of North Carolina-Chapel Hill Institutional Animal Care and Use Committee (IACUC). Approximately 10^9-10^{10} trypanosomes were isolated from infected rats. Trypanosomes were isolated from blood by centrifugation to separate plasma and red blood cells. Trypanosomes were found in the buffy coat, which was removed and combined with an equal volume of phosphate saline glucose (PSG) pH 7.4. This solution was passed through a DEAE column to separate white blood cells and any contaminating red blood cells from trypanosomes. Due to charge differences, trypanosomes passed through the column while blood components were retained on the column. Isolated trypanosomes were washed twice in PSG buffer.

Trypanosome Lysis. Trypanosomes were resuspended in lysis buffer with Complete proteinase inhibitors (Roche) and lysed in a nitrogen cavitation bomb. A solution of trypanosomes was held under 1000 psi nitrogen for 30 min to lyse cells. Lysis was visualized by viewing an aliquot of the cell suspension with a light microscope.

Lysis by nitrogen cavititation was the best lysis method, but other lysis methods were attempted before nitrogen cavitation. These methods included lysis by manual homogenization, sonication, and vortexing. Isolation procedures developed for acidocalcisomes (23) called for the use of silicon carbide and a mortar and pestle. Briefly the trypanosome pellet was mixed with silicon carbide, and trypanosomes were ground on ice using a mortar and pestle. After lysis, silicon carbide was removed from the lysed cellular components by a low speed centrifugation.

Another method used for lysis was manual homogenization. Trypanosomes were resuspended in lysis buffer and lysed with a Dounce homogenizer. Alternatively, a Tissuemiser or probe sonicator (Fisher Scientific) was used to lyse trypanosomes. The final method used was to mix trypanosomes with glass beads and vortex for several minutes.

Acidocalcisome Isolation. Acidocalcisomes were isolated using a lysis buffer containing 0.125 M sucrose, 50 mM KCl, 4 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, and 20 mM HEPES, pH 7.4 (23). Trypanosomes were lysed in nitrogen cavitation bomb as described above. Lysed trypanosomes were centrifuged at 900 rpm for 10 min to pellet cell debris. The supernatant was decanted and centrifuged at 1500 rpm for 10 min. The supernatant was decanted and saved, and the pellet resuspended in fresh lysis buffer (10 ml) and centrifuged at 1500 rpm for 10 min. The two supernatants were combined and centrifuged at 10000 g for 30 min in a Beckman Ultracentrifuge. The pellet was resuspended in 2 ml of lysis buffer by passage through a 22 gauge needle, and loaded on top of an Optiprep gradient (24/28/34/37/40% Optiprep in lysis buffer). The gradient was centrifuged at 50000 g for 1 h in a Beckman Ultracentrifuge. Acidocalcisomes were pelleted to the bottom. Fractions were collected from the top into 2 ml centrifuge tubes. The bottom 2 fractions and resuspended pellet were analyzed for the presence of acidocalcisomes.

Isolation of Other Organelles. The isolation of mitochondria and kinetoplast, nucleus, glycosomes and lysosomes were attempted.

Kinetoplast/Mitochondria Isolation. This isolation procedure was modified from previously published reports (19). Trypanosomes were suspended in a lysis buffer containing 50 mM HEPES-NaOH buffer (pH 7.2) containing 0.27 M sucrose, 1.0 mM EDTA, 1.0 mM $MgCl_2$, and a Complete protease inhibitor tablet (Roche). Trypanosomes were lysed as described

previously, and mitochondria were isolated. Residual cell debris was removed by a 900 g centrifugation at 4 ºC. The supernatant was centrifuged for 10 min at 12000 g to pellet crude mitochondria. The pellet was resuspended in 10 mM potassium phosphate buffer (pH 7.5) containing 0.25 M sucrose and 1.0 mM EDTA, and was centrifuged again at 12000 g for 10 min. Isolated mitochondria were resuspended in a small amount of buffer.

Lysosome and Glycosome Isolation. Trypanosomes were resuspended in SHK buffer (250 mM sucrose, 50 mM HEPES, 25 mM KCl, pH 7.4), containing protease inhibitors. Trypanosomes were lysed in a nitrogen cavitation bomb as described above. After lysis, 1 mM EDTA was added to the buffer containing trypanosomes. Cell debris was pelleted at 700 g for 10 min to yield crude nuclear pellet. The supernatant was clarified by centrifuging at 2800 g for 10 min to produce a large granule fraction (LG). The supernatant was then centrifuged at 15000 g for 10 min to yield a small granule fraction (SG). Optionally, the post SG supernatant could be centrifuged at 123000 g for 90 min in a 50.2 Ti rotor. This centrifugation produced a microsomal pellet fraction and soluble supernatant. Otherwise, the LG and SG fractions were further fractionated by isopycnic centrifugation in Percoll. LG and SG fractions were made 57.6% with respect to Percoll in SHKE and layered underneath a discontinuous gradient (43.2/28.8/20.3%) and centrifuged for 30-35 minutes at 25000 rpm in SW41 or SW28 rotor (depending on volume of supernatant). Fractions banding between the 28.8/43.2% and 43.2/57.6% interfaces were made 57.6% with respect to Percoll again and centrifuged again through the same gradient. Fractions on top of and in the 20.3% Percoll layer (crude flagella) were adjusted to 28.8 % Percoll and layered under a discontinuous gradient containing 20.3/17.3/14.4% Percoll in SHKE buffer. This was centrifuged again as above to obtain the lighter membrane fractions. The lysosomes are at

the 28.8/43.2% interface and the glycosomes are at the 43.2/57.6% interface after the second centrifugation. This isolation procedure was modified from a method published by Grab et al (11).

Nuclei Isolation. Trypanosomes were lysed as described above. Lysed cells were underlaid with 10 ml of 0.3 M sucrose in 8% poly vinyl pyrolidine (PVP) plus Complete protease inhibitors, 5 μL of 1 M DTT, and centrifuged at 11000 g, at 4°C for 20 min as described previously (21). The crude nuclear pellet was immediately resuspended with 1 min bursts of the Tissuemiser in a total volume (per 2 X 10^{10} cells) of 8 ml of 2.1 M sucrose in 8% PVP, 50 μL of 1 M DTT, protease inhibitors, and 0.05% Tween 20. Approximately 12 ml of this solution was loaded per SW28 tube containing a discontinuous step gradient (from the bottom, 8 ml of 2.3 M sucrose/PVP, 8 ml of 2.1 M sucrose/PVP, 8 ml of 2.01 M sucrose/PVP, all containing protease inhibitors). For the preparation to be successful, it is essential that no more than 2.5 X 10^{10} cell equivalents be loaded into each SW28 tube. The gradient was centrifuged at 100000 g, in a SW28 rotor for 3 hours at 4°C and immediately unloaded from the top. Fractions were examined by phase contrast microscopy. Nuclei were recovered at the 2.1/2.3 interface. Quantification of nuclei was performed by optical density at 260 nm; for *T. brucei*. OD_{260nm} corresponds to ~ 10^8 nuclei and has an $OD_{260\ nm}/OD_{280\ nm}$ ratio of ~1.3.

Alternatively, instead of PVP, sucrose solutions were used in buffer. Briefly, to the final homogenate, 10 ml of 2.3 M sucrose in Tris buffer (containing 0.75% Triton X-100) was added. This was placed on top of 2.3 M sucrose in Tris buffer in a 25 X 89 mm centrifuge tube and centrifuge at 40500 g in a SW28 rotor for 70 m at 4°C to pellet nuclei. This method was adapted from a cancer cell nuclei isolation procedure (9).

TEM Analysis of fractions. Samples were analyzed using a LEO EM 910 Transmission Electron Microscope (TEM). Acidocalcisomes samples were mounted on formvar coated grids and allowed to dry. This was done to retain electron density of the organelles (7). Samples were viewed using 8000 – 100000 magnification. Other organelle fractions investigated were fixed in 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer (Fisher Scientific), and were embedded and sectioned by personnel at the Microscopy Services Lab at UNC.

Results and Conclusions

The acidocalcisomes and mitochondria were isolated using protocols adapted from previous work (23). Of all the lysis methods attempted, the use of a nitrogen cavitation bomb to disrupt the cellular membranes worked the best. Other methods, including sonication and the use of various homogenizers, did not completely lyse cells. For many of the methods attempted, many trypanosomes survived lysis attempts with motility intact.

Acidocalcisomes were analyzed using TEM to visualize electron density of the organelles (Figure 1). Low and high resolution micrographs show electron dense particles, which are relatively pure (no contaminating organelles or debris). The organelles were approximately 50 nm in size, consistent with previous data (7). The fraction also reacted with an antibody raised against yeast vacuolar H+ ATPase 69 kDa subunit (data not shown).

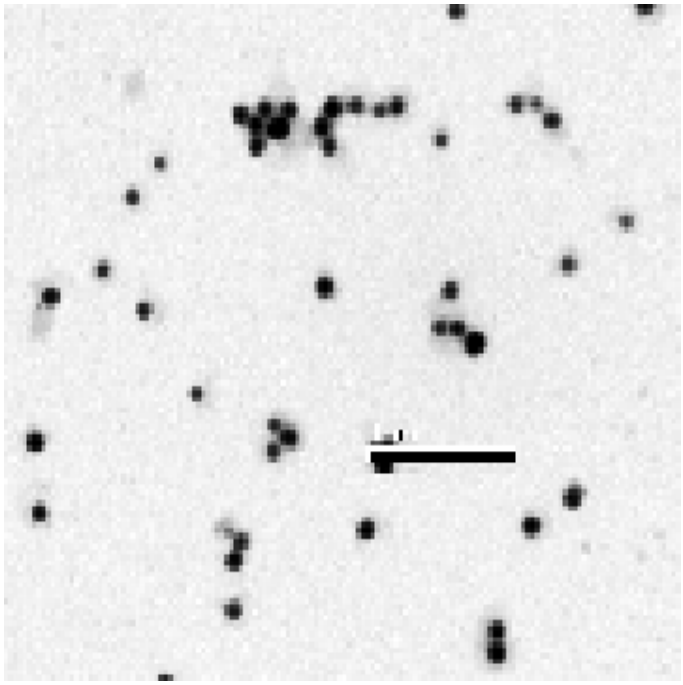
Isolation procedures for the acidocalcisomes were successful. However, due to a lack of availability of antibodies raised against *Trypanosoma brucei* organelles, the purity of the isolated fraction was not determined. The isolated fraction did not cross react with an antibody raised against lysosome proteins (anti-p67), and was therefore free of lysosomal contamination (data not shown).

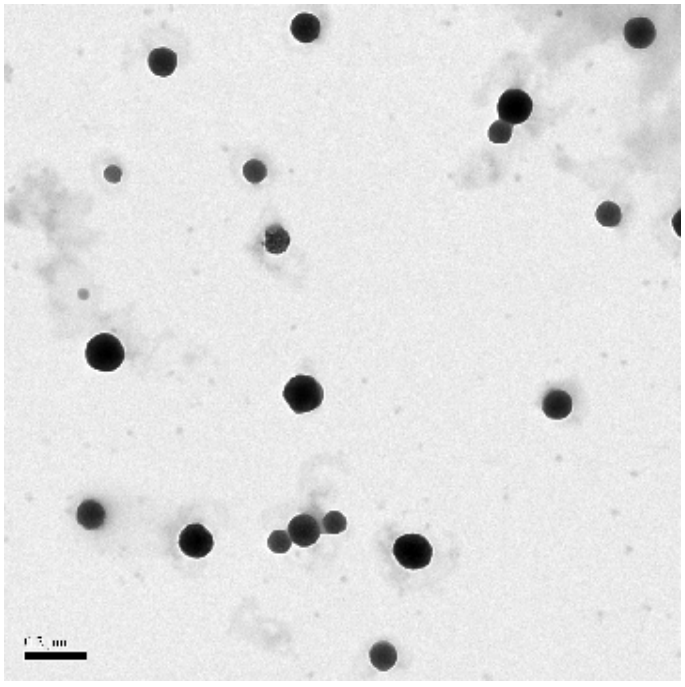
Although it was proven in Appendix I that DB75 accumulated in the acidocalcisomes, before quantification of the relative amount of the compound in the organelle can be performed, purity of the fraction must be determined. Additionally, other organelles in the trypanosomes must be isolated in order to determine mass balance of diamidines in trypanosomes. Isolation of the nucleus has been a particular problem, with contamination from unbroken cells, flagella and membranes being an issue. Additionally, using optical density methods, no purification of nuclei was seen (data not shown). Another technique to

determine DNA associated concentrations could be to isolate total cellular DNA and separate it into kinetoplast and nuclear fractions. For the lysosomes and glycosomes, preliminary evidence suggests that the organelle fractions do contain lysosomes, but cross-reactivity with antibodies from other organelles has not been attempted yet. It may be necessary to use enzyme assays to determine relative purity of each organelle fraction. Enzyme assays for many organelles have been published (24).

Although only one organelle has been successfully isolated, this is an excellent first step towards isolating all organelles in trypanosomes that accumulate DB75 and related compounds. It is estimated that with modifications to the other procedures, isolation of the other organelles will be successful. Then quantification of compound accumulation and further studies investigating mechanism of action can be undertaken.

Figure 1: Low and high magnification micrographs of isolated acidocalcisomes. Low magnification (8,000X), high magnification (25,000X).





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