Biological and Pharmacological Investigations of Novel Diamidines in Animal Models of Human African Trypanosomiasis

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John Thuita Kibuthu

aus Nairobi Kenya

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Prof. J. Schibler, Dekan

To my wife Faith and our
daughters Doreen and Sonia

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Abbreviations

AUC	Area under the curve
BBB	Blood brain barrier
BID	Dosing twice a day
Cmax	Maximum concentration
CNS	Central Nervous system
CPDD	Consortium for Parasitc Drugs Development
CSF	Cerebrospinal fluid
DPI	Days post infection
DPT	Days post treatment
EoT	End of treatment
HAT	Human African trypanosomiasis
HPLC	High performance liquid chromatography
IM	Intramuscular
IV	Intravenous
LDD	Last drug dose
MS	Mass spectrometry
NECT	Nifurtimox eflornithine combination therapy
NGOs	Non-governmental organizations
NTDs	Neglected tropical diseases
NTDs	Neglected tropical diseases
PK	Pharmacokinetics
QD	Dosing once a day
Swiss TPH	Swiss Tropical and Public Health Institute
TK	Toxicokinetics
Tmax	Time to maximum concentration
ToC	Test of cure
TRC-KARI	Trypanosomiasis Research Centre Kenya Agricultural Research Institute
VAT	Variable antigenic types
VSG	Variant surface glycoproteins
BSFs	Bloodstream forms
mAECT	mini-anion exchange centrifugation technique
WHO	World Health Organization
PTRE	Post treatment reactive encephalopathy
ISG	Invariant surface glycoprotein
PPPs	Public Private Partnesrships
IC_{50}	Inhibitory concentration 50
MTR	Median time to relapse

Summary

African sleeping sickness, also called human African trypanosomiasis (HAT), results from the infection of humans with either of two protozoan parasites, *Trypanosoma brucei gambiense* and *T. b. rhodesiense*. HAT is transmitted by tsetse flies (*Glossina spp*) and, like the vector, is found exclusively in Africa between the latitudes 14° North and 29° South. A total of 50 million people live in foci where active transmission is possible and are therefore at risk of infection; however, the annual incidence and estimated prevalence currently stand at 7139 and 30 000 cases respectively. When trypanosomes are inoculated into a human host, the resulting clinical disease is classified into a first (early) stage in which trypanosomes are localised within the haemo-lymphatic system and a second (late) stage in which trypanosomes have crossed the blood brain barrier (BBB) and invaded the central nervous system (CNS).

Currently, pentamidine and suramin are used to treat the first stage of *T. b. gambiense* and *T. b. rhodesiense* HAT, respectively. On the other hand, eflornithine and the nifurtimox eflornithine combination therapy (NECT) are the preferred treatments for second stage *T. b. gambiense* HAT. The organo-arsenic drug melarsoprol may be used for both forms of HAT but is mainly used against *T. b. rhodesiense*. Clearly, the therapeutic options for HAT are very limited. In addition, available drugs are associated with different levels of toxicity, especially melarsoprol which causes a post treatment reactive encephalopathy (PTRE) in 5-10% of treated patients, up to 50% of PTRE patients may die. There are also reports of high melarsoprol treatment failure rates in some foci and there is a lack of easy to use oral formulations for all the drugs. We have carried out biological and pharmacological investigations of potential new drug candidates in animal models of HAT with the objective of contributing to the development of safe, efficacious and easy to use treatments for HAT. The studies were carried out in the context of a PhD programme at the Swiss TPH/University of Basel and were anchored onto an ongoing diamidines development project of the Consortium for Parasitic Drug Development (CPDD). Vervet monkeys (*Chlorocebus [Cercopithecus] aethiops*) were the main model for this study.

To prepare for the studies in monkeys, one prodrug (DB289) was evaluated in mouse models of first stage HAT. We obtained good activities against different trypanosome isolates, including the one that is used in the monkey model, *T. b. rhodesiense* KETRI2537. We further evaluated the metabolism of the prodrugs in monkey liver microsomes. In all cases, prodrugs were metabolized to generate expected intermediate and active metabolites, thus allowing us to proceed

to test the compounds for safety in un-infected monkeys. We determined that in monkeys: i) diamidine toxicity was dependent on the dose and duration of dosing, ii) the plasma concentrations of active metabolites were potentially therapeutic for HAT, and iii) the dose level at which there were no observed adverse effects (NOAEL). Three prodrugs (DB289, DB844 and DB868) and one active compound (DB829) were subsequently evaluated for efficacy at dose rates that were equal or below NOAEL. In general, the prodrugs were highly active against first stage HAT after oral administration and one prodrug (DB844) had additionally an improved activity (43%) in the second stage monkey HAT model in comparison with pentamidine (0%). The intramuscularly administered parent compound DB829 was fully curative in the second stage HAT model at 2.5 mg/kg x 5 days.

Our findings suggest that the two compounds (oral DB868 and intramuscular DB829) should be recommended to enter the regulatory phase of development as potential HAT drugs. Oral DB868 cured the first stage HAT model at a daily dose of 3 mg/kg for 7 days (cumulative dose, CD = 21 mg/kg) compared to a maximum tolerated daily dose of 30 mg/kg for 10 days (CD = 300 mg/kg). The efficacy, safety and pharmacokinetic profiles suggest that this compound would be a useful clinical candidate using an optimal dosing duration of 5-7 days. The second compound, intramuscular DB829, cured the second stage HAT model at a daily dose of 2.5 mg/kg for 5 days and was tolerated at 5 mg/kg for 5 days (CD = 25 mg/kg). Pharmacokinetic analysis indicated the intramuscular administration of DB829 resulted in better systemic bioavailability, thus accounting for the improved efficacy in comparison with oral dosing.

Zusammenfassung

Die Afrikanische Schlafkrankheit, auch humane Afrikanische Trypanosomose (HAT) genannt, wird im Menschen durch eine Infektion mit einem der zwei einzelligen Parasiten, *Trypanosoma brucei gambiense* und *T. b. rhodesiense* verursacht. HAT wird durch Tsetsefliegen (*Glossina* spp) übertragen und kommt wie der Vektor ausschließlich in Afrika zwischen den Breitengraden 14° Nord und 29° Süd vor. Insgesamt 50 Millionen Menschen leben in den Gebieten, in denen eine aktive Übertragung möglich ist, und sie sind somit dem Risiko einer Infektion ausgesetzt. Die Inzidenz lag 2010 bei 7139 gemeldeten Fällen und die Prävalenz wurde auf 30 000 Fälle geschätzt. Wenn Trypanosomen in einen menschlichen Wirt gelangen verläuft die Erkrankung in zwei Stadien. In einer ersten (akuten) Phase sind die Trypanosomen im hämo-lymphatischen System lokalisiert, in der zweiten (chronische) Phase überwinden die Trypanosomen die Blut-Hirn-Schranke und infizieren das zentrale Nervensystem (ZNS).

Derzeit werden Pentamidin und Suramin verwendet, um das erste Stadium einer *T. b. gambiense* Infektion respektive einer *T. b. rhodesiense* Infektion zu behandeln. Eflornithin und die Nifurtimox-Eflornithin Kombinationstherapie (NECT) sind die bevorzugten Medikamente für das zweite Stadium einer *T. b. gambiense* HAT. Das arsenhaltige Medikament Melarsoprol kann für beide Formen von HAT verwendet werden, wird aber hauptsächlich nur bei der durch *T. b. rhodesiense* verursachten Schlafkrankheit verwendet. Die therapeutischen Möglichkeiten zur Behandlung von HAT sind eindeutig begrenzt. Zusätzlich sind die verfügbaren Medikamente in unterschiedlichem Masse toxisch. Insbesondere Melarsoprol kann bei 5-10% der behandelten Patienten eine reaktive Enzephalopathie (PTRE) verursachen, die in 50% der Fälle tödlich ist. Zusätzlich gibt es Berichte von Behandlungsmisserfolgen mit Melarsoprol, in manchen Schlafkrankheitsgebieten bis zu 30%. Die bisher verfügbaren Medikamente müssen intramuskulär oder intravenös verabreicht werden. Es fehlen leicht verabreichbare orale Formulierungen. Mit dem Ziel, eine verträgliche, wirksame und leicht zu verabreichende Medikation gegen HAT zu finden, haben wir biologische und pharmakologische Untersuchungen von potentiellen neuen Wirkstoffkandidaten in Tiermodellen durchgeführt. Die Studien waren Teil eines laufenden Diamidin-Entwicklungsprojekts des „Consortium for Parasitic Drug Development“ (CPDD) und wurden im Rahmen einer Doktorarbeit am Swiss TPH / Universität Basel durchgeführt. Meerkatzen (*Chlorocebus [Cercopithecus] aethiops*), eine Affenart, waren das Hauptmodell für diese Arbeit.

Während der Vorarbeit, wurde eine Substanz (DB289), ein Prodrug, in Mausmodellen mit dem ersten Krankheitsstadiums untersucht. Die Substanz zeigte gute Aktivitäten, einschliesslich gegen den Parasitenstamm *T. b rhodesiense* KETRI2537, der ebenso im Affenmodell verwendet wird. Zusätzlich haben wir die Metabolisierung der verschiedenen Prodrugs in Affen-Lebermikrosomen untersucht. In allen Fällen wurden die Prodrugs wie erwartet in Zwischen-und aktive Metabolite umgesetzt. Die Ergebnisse ermöglichten es uns, die Verträglichkeit der Substanzen in uninfizierten Affen zu prüfen. Wir stellten fest, dass in Affen: i) die Toxizität der Diamidine von der Dosis und der Dauer der Dosierung abhängig war, ii) die Plasma-Konzentrationen der aktiven Metaboliten potentiell therapeutisch für HAT sind und konnten iii) die Dosis bestimmen, die keine erkennbaren und keine messbaren Schädigungen hinterlässt (NOAEL: no observed adverse effect level). Die drei Prodrugs DB289, DB844 und DB868 und das aktive Diamidin B829, wurden anschließend auf ihre Wirksamkeit untersucht. Die verwendete Dosis war entweder gleich oder unterhalb NOAEL. Im Allgemeinen waren die Prodrugs hochaktiv gegen das erste Schlafkrankheitsstadium bei einer oralen Verabreichung. Eine Substanz (DB844) wies im Vergleich zu Pentamidin (0%) zusätzlich eine verbesserte Aktivität (43%) im chronischen Affenmodell (mit ZNS- Infektion) auf. Das intramuskulär verabreichte Diamidin DB829 konnte bei einer täglichen Dosis von 2.5 mg/kg für 5 Tage das chronische Affenmodell vollständig heilen.

Unsere Ergebnisse legen nahe, dass die beiden Wirkstoffe (DB868 oral und DB829 intramuskulär) als potenzielle HAT Medikamente weiterentwickelt werden sollten. Oral verabreichtes DB868 heilte das akute HAT Affenmodell bei einer täglichen Dosis von nur 3 mg/kg und einer 7 Tage Behandlung (kumulative Dosis, CD = 21 mg/kg). Im Vergleich dazu lag die maximal tolerierte Dosis von DB868 bei 30 mg/kg für 10 Tage (CD = 300 mg/kg). Die Wirksamkeit, Verträglichkeit und das pharmakokinetische Profil zeigen, dass diese Verbindung ein guter klinischer Kandidat für das erste Stadium sein könnte, mit einer optimalen Dosierungsdauer von 5-7 Tagen. Die zweite intramuskulär verabreichte Verbindung DB829, heilte das zweite Krankheitsstadium im Affenmodell bei einer täglichen Dosis von nur 2,5 mg/kg und einer 5 Tage Behandlungsdauer. Die tolerierte Dosis lag bei 5 mg/kg für 5 Tage (CD = 25 mg/kg). Die pharmakokinetische Analyse zeigte, dass die intramuskuläre Verabreichung von DB829 zu einer verbesserten systemischen Bioverfügbarkeit führt und damit zur verbesserten Wirksamkeit im Vergleich zur oralen Dosierung.

Chapter 1: General Introduction

The Problem of Human African Trypanosomiasis (HAT)

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a debilitating disease that is caused by two flagellated protozoan parasites, *Trypanosoma brucei (T. b.) gambiense* and *T. b. rhodesiense*. The two pathogens are native to the African continent. Not surprisingly, Africa has borne the blunt of the losses attributable to sleeping sickness since only a limited number of infections have been diagnosed in tourists or travellers from other countries visiting areas where active disease transmission was occurring (Sinha *et al*., 1999; Jelinek *et al.*, 2002; Moore *et al*., 2002). HAT is ranked among the thirteen most neglected tropical diseases (NTDs), with disability adjusted life years (DALY's) lost of 1 673 000 years (Vanderelst and Speybroeck, 2010). NTDs have in common, a lack or shortage of efficacious medicines that are also safe to use, easily administrable and inexpensive, in order to benefit patients in resource-poor settings where NTDs are common.

HAT is transmitted by tsetse flies (*Glossina* spp), and as a result, is only found between latitudes 14° North and 29° South, the ecological limits of the vector. Tsetse flies also transmit animal pathogenic trypanosomes that together with their vectors, cover an estimated area of 10 400 000 km^2 of agricultural land and are estimated to cost Africa an annual income of 4.5 billion USD (Wilson *et al*, 1963; DFID, 2010; Tesfaye *et al*., 2012). Indeed, successive African Governments from colonial times and the African Union (AU) have acknowledged that human and animal trypanosomiasis need to be controlled to improve health and economic development of Africa (Wilson, 1963; Simarro *et al*., 2008; African Union, 2002).

A total of 36 countries are classified by the World Health Organization (WHO) as endemic for HAT (Simarro *et al*, 2008; WHO, 2012a). A number of these countries have however not reported a single case of the disease in more than 10 years (WHO, 2012a) and according to Stuart *et al*., (2008) HAT is a significant public health problem in only 20 countries. However, this restricted number of countries may mask the fact that the disease is a threat to an estimated 50-60 million people who live in foci where active transmission is possible (Stuart *et al*., 2008; WHO, 2012a). The significance of this threat is amplified by the fact that a majority of the affected people are exposed to the tsetse fly menace in the course of eking out a livelihood in agriculture, fishing,

animal husbandry or hunting (Simarro *et al*., 2008; WHO, 2012a). Preventing new infections is therefore a formidable challenge indeed.

HAT occurs in cycles of epidemics interspersed with periods when the disease was almost decimated (Figure 1). During the most recent epidemic which peaked in 1998, a total of 37, 991 new cases of HAT were reported to the World Health Organization (WHO), with an estimated prevalence of 300,000 to 500,000; these high numbers were considered to be the result of an underestimation of the true situation because of insufficient coverage by surveillance systems (Chappuis *et al*., 2010; WHO, 2012a). The extent of this human catastrophe was magnified by the fact that only an estimated 10-20% of infected persons are accessed by disease control authorities and put on treatment (Torreele *et al*., 2010; WHO, 2012a). Without treatment, it is widely accepted that HAT is invariably fatal (Kennedy 2004; WHO, 2012a).

Yet sleeping sickness has been shown to be amenable to therapy. In the recent 1998 epidemic, National disease control authorities were galvanised into action, supported by WHO and non-governmental organizations (NGOs) such as Medicines San Frontiers, philanthropic organizations such as Bill and Melinda Gates Foundation and bilateral donors (WHO, 2012a). The support was channelled to strengthening of disease surveillance activities, efficient supply of existing drugs and increased drug development activities (WHO, 2012a; Paine *et al*., 2010; Chappuis *et al.,* 2010). The re-invigorated activities led to a major decline in HAT incidence, which by 2010, stood at 7, 139 cases, which was an 81% decline in comparison to the 1998 figures. The prevalence has similarly declined to an estimated 30, 000 cases, showing that, HAT is clearly in retreat.

Unfortunately, the optimistic prediction that HAT could soon be eliminated as a disease of public health significance throughout Africa (Simarro *et al.,* 2011) could yet prove to be a mirage. The trypanosome has shown itself to be quite resilient and is capable of exploiting weaknesses in disease surveillance and/or unavailability of the right tools (drugs and diagnostics) to make an unwanted comeback. Historical data (Figure 1) capture this succinctly. In addition, one Belgian and one German tourist came down with sleeping sickness recently after visiting Maasai Mara Game Reserve in Kenya, an area not regarded as an active HAT focus (Clerix *et al*., 2012; Wolf *et al*., 2012); these cases serve as a portent reminder that the danger of HAT flare ups remains.

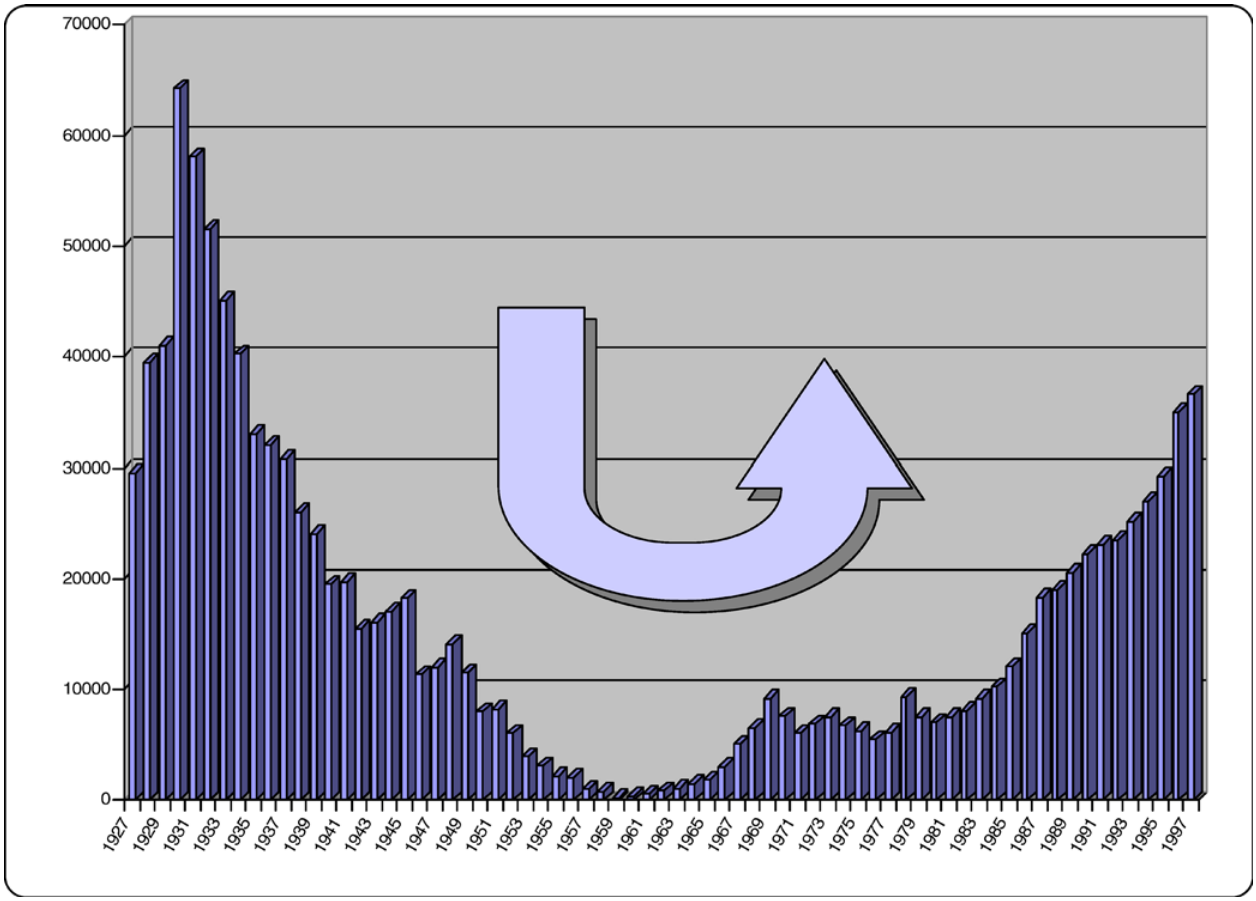

Figure 1: Human African trypanosomiasis from 1927 to 1998 (Source: Simarro *et al*., PLoS Med 2008)

The Parasite

Taxonomy

Trypanosomes were first observed in a trout in 1841. The genus name *Trypanosoma* was however coined in 1843 when morphologically similar protozoan parasites were detected in the blood of a frog (WHO, 2012b). Trypanosomes were later confirmed to be pathogenic and to be the aetiological agents of surra in horses and nagana in cattle before being identified in a human patient with recurrent fevers in the Gambia in 1901 (WHO, 2012b). They were later confirmed to be the aetiological agent of sleeping sickness, then known as sleepy distemper or African lethargy (WHO, 2012b).

Trypanosomes are classified under Kingdom Protista, sub-Kingdom protozoa, Phylum Sarcomastigophora and Class Mastigophora although there is some difference of opinion among taxonomists on exact application of these terms (Hoare, 1972). Trypanosomes are then placed into the order Kinetoplastida, family Trypanosomatidae and genus Trypanosoma (Hoare, 1972). Genus Trypanosoma is further subdivided into two sections, Salivaria and Stercoraria. Salivarian trypanosomes mature in the salivary medium of the "anterior station" and are transmitted by inoculation into susceptible vertebrate hosts (Hoare 1972); the exact location of this maturation is in the salivary glands (e.g. *T. brucei* spp) or in the proboscis (*T. congolense* and *T. vivax*) (Hoare, 1972; Vickerman, 1985; Peacock *et al*, 2012). Section Salivaria consists of four subgenera, including *Trypanozoon* (*T. brucei brucei*, *T. b. rhodesiense*, *T. b. gambiense*, *T. evansi* and *T. equiperdum, T. equinum), Duttonella* (*T. vivax* and *T. uniforme)*; *Nannomonas* (*T. congolense* and *T. simiae)* and *Pycnomonas* (*T. suis*) *(*Hoare 1972). Apart from *T. b. rhodesiense* and *T. b. gambiense* which cause disease in humans, other members of section Salivaria cause disease in livestock and wildlife. Domestic and wild animals have further been identified as reservoirs of the zoonotic *T. b. rhodesiense* (Simarro *et al*., 2008) and less commonly of *T. b. gambiense* (Cordon–Obroso *et al,* 2009); *T. b. gambiense* is mainly assumed to be anthroponotic (Simarro *et al*, 2011; WHO, 2012a).

Section Stercoraria has only one pathogenic trypanosome, *T. cruzi*, which causes Chagas disease (American trypanosomiasis). *T. cruzi* matures in the faecal medium of the "posterior chamber" of the Triatomine insect, also called reduviid or kissing bug, and is therefore transmitted through contamination of bite wounds with the insect's faeces (Hoare, 1972). *T. cruzi* infections are prevalent in Latin America with a prevalence of 12-16 million cases (Diaz, 2009)

Life Cycle

The life cycle of trypanosomes involves two hosts, a mammal which acts as the definitive host and an insect vector which acts as the intermediate host (Vickerman, 1985; http://content.lib.utah.edu:81/cgi-bin/showfile.exe?CISOROOT=/EHSL-NOVEL&CISOPTR=426&filename=900.pdf). In an infected vertebrate, the dividing trypanosome population are the long slender trypomastigotes, also called long slender bloodstream forms (BSFs). The long slender BSFs divide rapidly by binary fission, with a doubling time of approximately 6-8 hours (Vickerman, 1985). For their metabolic needs, BSFs rely exclusively on glucose which is freely and abundantly available in host blood. As a result, BSFs obtain their energy by glycolysis which occurs in spherical glycosomes. They have no capacity for cytochrome mediated terminal respiration since their tricarboxylic acid cycle (Krebs cycle) is silenced. The BSFs multiply unchallenged until the host mounts an immune (IgM) response against the predominant variant antigen type (VAT) which is then quickly decimated. However, this does not lead to cure, rather it allows another previously minor VAT to assume the dominant position and generate the next wave of parasitaemia. The different VAT types are generated through the process of antigenic variation that is encoded in trypanosome genome by up to 1000 genes. Thus, parasitaemia and associated trypanosome-induced host tissue pathology continue until terminated by death of the host or therapeutic intervention. Some authors however argue that the evolution of trypanosome infections could be ameliorated or stopped altogether through the action of host immune systems, a process called trypanotolerance. Trypanotolerance is well documented for cattle breeds such as the Orma Boran and Ndama (Grace Murilla, personal communication). It may also occur in some human *T .b. gambiense* infections (Chechi *et al*., 2008) although most authors support the view that HAT is invariably fatal unless patients are treated (Kennedy, 2004, Kuepfer *et al*., 2011). During peak and declining parasitaemia, a new morphological form, the short stumpy trypomastigote or short stumpy BSF is generated. These short stumpies are the tsetse fly infective forms.

The short stumpy BSFs are picked up when the flies take a blood meal from an infected mammal. The blood is swallowed to the fly's midgut where the short stumpy BSFs transform to procyclics while within the peritrophic membrane (endoperitrophic space) (Vickerman 1985). The transformation to procyclics is characterised by changes in morphology (elongation and loss of the VSG coat) and a biochemical switch from dependence on glucose to dependence on the amino acid proline for metabolism. This clever tactical switch by the trypanosome is necessitated by the fact that glucose is unavailable in tsetse flies which, instead of glucose, depend on proline for energy

metabolism. Furthermore, procyclics adapt to a cytochrome mediated terminal respiration by enlarging and activating their mitochondria (Vickerman, 1985). The procyclics invade the ectoperitrophic space within 4 days of an infective blood meal, multiply rapidly and subsequently migrate to the proventriculus. In the proventiculus, the procyclics transform to mesocyclics, re-invade the endotrophic space and migrate to the salivary glands via the oesophagus, mouthparts and salivary ducts (Vickerman, 1985). In salivary glands, trypanosomes change to epimastigotes which proliferate while attached to the salivary gland epithelia. Finally, epimastigotes transform to premetacyclics and then to metacylics which acquire a variant surface glycoprotein (VSG) coat, thus acquiring the ability to survive in mammals. It takes 20-30 days for mature mammal infective metacyclics to be generated. When tsetse flies feed on a new host, the metacyclics are deposited in dermal or subcutaneous tissues where they rapidly transform to long slender trypomastigotes, thus completing the cycle.

The trypanosome life cycle reveals the different strategies employed by trypanosomes against host defences. In tsetse flies, evasion: procyclics avoid migrating through the haemolyph, which contains anti-trypanosomal agents such as atacin and defensin. Despite this evasion tactic, many tsetse flies are still able to free themselves of infecting trypanosomes, which partly explains why trypanosome infection rates are only estimated at 0.1% (Vickerman, 1985). In mammals, the variant surface glycoprotein coat (VSG), which is attached to the plasma membrane via GPI anchors, serves as physical barrier against the innate immune system while antigenic variation enables the pathogen to survive the attacks of humoral immune system. The VSG coat covers the entire trypanosome with the possible exception of the flagellar pocket through which receptor mediated endocytosis of proteins and protein-drug complexes (e.g. for suramin) are thought to occur (Fairlamb and Bowman, 1980). The trypanosome defence against natural mammalian immune system is so effective that the mammals require assistance to be freed of the trypanosome invaders.

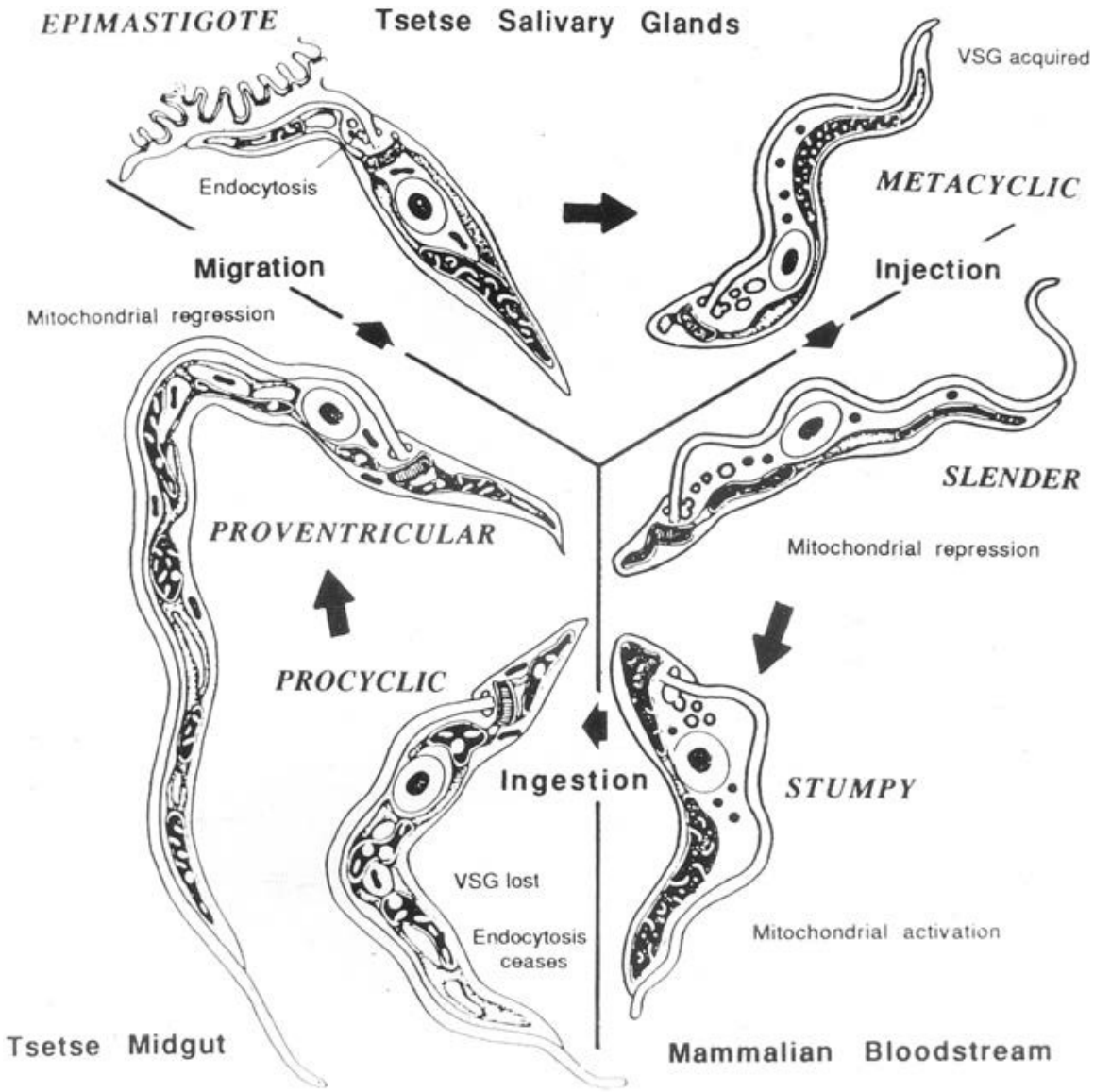

Figure 2: Development cycle of *Trypanosoma brucei*. Source: Hadjuk *et al*., Am J Med Sci 1992

Clinical Signs, Diagnosis and the Disease in Animal Models

After the infective tsetse bite, a local inflammatory swelling (chancre) develops at the site of the fly bite within 5-15 days (Kennedy 2004; Sternberg *et al*., 2006). Trypanosomes establish infection in the haemo-lymphatic system, marked by general malaise, fluctuating fever and general lymphadenopathy. The enlargement of lymph nodes of the neck region (Winterbottom's sign), is commonly observed in *T. b. gambiense* infection. Other clinical signs that may be observed during the 1st (early, haemolymphatic) stage of HAT include pale mucous membranes indicative of anaemia, headache, fever, pruritus, oedema, splenomegaly, hepatomegaly, and weight loss (Moore *et al.,* 2002; Kennedy, 2004). Clinical signs of 2nd (late, CNS) stage of HAT include altered sleep patterns, sensory disturbances, abnormal movements, limb tremors, muscle fasciculation, mental changes or psychiatric disorders, anorexia, coma and finally death (Kennedy, 2004).

Diagnosis of HAT relies mainly on visualization by microscopy of the causative trypanosome parasites in stained smears of body fluids such as blood, cerebrospinal fluid (CSF) and lymph node aspirates (WHO, 1998; Kennedy, 2004). Microscopy has limited sensitivity due to low and fluctuating parasitaemias in HAT patients (Van Meirvenne, 1999). Specifically, examination of wet smears of blood by the matching method of Herbert and Lumsden (1976) can in theory detect up to 10,000 trypanosomes/ml of blood. In practice, however, observation of one trypanosome in 20 microscopic fields corresponds to a parasitaemia of 250,000 trypanosomes/ml of blood (equivalent to antilog 5.4) and it is unlikely laboratory personnel will routinely examine more than 20 microscope fields per sample. The most sensitive microscopic techniques include the mini-anion exchange centrifugation technique (mAECT) and haematocrit centrifugation technique (HCT) also called capillary tube centrifugation technique or buffy coat technique (Woo, 1970; Lumsden *et al*., 1979; Cattand *et al.,* 1988) for blood and the modified single centrifugation or double centrifugation techniques for CSF (Miezan *et al.,* 2000). The centrifugation component in these techniques improves their sensitivity to approximately 1000 trypanosomes/ml of blood. In areas that are endemic for *T. b. gambiense* HAT, a serological test, the card agglutination test for trypanosomiasis (CATT) is routinely used as a screening test before microscopy (Simarro *et al.,* 2008).

Animal models of HAT have been used to study different aspects of the disease including chemotherapy, pathogenesis, and pathology and to evaluate the potential of resultant host clinical and biological changes as diagnostic and disease staging markers (Jennings *et al.,* 1977; Fink *et al.,*

1983; Gichuki and Brun, 1999; Kagira *et al*., 2006). These models are based on experimental infection of various animal hosts with *T. b. brucei*, *T. b. rhodesiense* or *T. b. gambiense*. Confirmation that these parasites are able to establish infections and characterization of the resultant disease progression in terms of clinical signs, immunology and pathology are some of the indicators of a well developed animal model. The most commonly used models for HAT research are the mouse, rat and other rodents, and vervet monkey models. Characterisation of these HAT models shows that the disease occurs in two stages irrespective of the host: a first stage in which trypanosomes are localised in the haemo-lymphatic system and a second stage in which the central nervous system (CNS) is invaded by trypanosomes, indicating that the basic pattern is similar to the disease in humans (Jennings *et al.,* 1977; Poltera *et al.,* 1980). The *T. b. brucei* GVR 35 model, in which mice are treated at 21 days post infection (Jennings *et al*., 1977), is widely used in murine chemotherapy trials of second stage HAT. The vervet monkey model is similarly widely used in preclinical drug research, with treatments for first stage compounds initiated at 7 days post infection while those for second stage compounds are initiated at 28 days post infection (Gichuki and Brun, 1999). The monkey model has the added advantage of permitting collection of cerebrospinal fluid (CSF) for monitoring CNS infections and has been described as closely mimicking the human disease in terms of clinical signs, immunological status and pathology (Schmidt and Sayer, 1982).

Taking the Fight to the Trypanosome

Vaccination, chemotherapy, patient and vector management are in general key strategies that are employed to fight vector-borne infectious diseases. The phenomenon of antigenic variation displayed by mammalian stages of trypanosomes however makes it very difficult, if not impossible, to develop vaccines based on their variant surface glycoproteins (VSG's) (Vickerman, 1985). The invariant surface glycoproteins on the trypanosome's plasma membrane are also not useful vaccine candidates since they are protected from contact with components of the host's immune system by the 12-15 nM thick VSG coat. However, there are better prospects of success in developing transmission blocking vaccines that interfere with the maturation of the insect stages of the trypanosome (Aksoy *et al*., 2001; Macleod *et al*., 2007). The tsetse flies can also be targeted through mass spraying with insecticides followed by the sterile insect technique to eradicate residual fly populations. Tsetse eradication campaigns have been successfully carried out in Zanzibar and Botswana and are in progress in three tsetse belts, Lake Victoria basin, Mwea/Meru and Lake Bogoria, in Kenya. The eradication activities are carried out under the leadership of the

Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) that was formed by African heads of state and Government in 2002. Indeed, the African Union believes that tsetse flies can actually be eradicated from the African continent (African Union, 2002). Currently, however, the fight against human infective trypanosomes, especially *T. b. gambiense* infection which constitute more than 95% of HAT cases (WHO, 2012a), relies heavily on surveillance and treatment of infected persons. The chemotherapy of HAT currently relies on the following drugs:

Pentamidine

Pentamidine was introduced for clinical use in 1941, becoming the first of its class to attain this milestone. Currently, 71 years later, it remains the only diamidine in clinical use and is recommended for the treatment of the 1st stage of *T. b. gambiense*-caused West African form of HAT (WHO, 2012a). The drug is available as pentamidine isethionate, Pentacarinat® (sanofi-aventis) in 200 mg vials for deep intramuscular injection at a dose rate of 4 mg/kg x 7 days qd (Burri, 2010). Relative to other HAT drugs, pentamidine is well tolerated. However it still causes non-negligible side effects including pain and/or transient swelling at the injection site, abdominal pain, diarrhoea, nausea, vomiting and hypoglycaemia (Burri, 2010). Other less commonly encountered adverse reactions include QT-prolongation and hyperglycaemia (Burri, 2010). Available mechanistic data show that pentamidine is selectively accumulated by trypanosomes via the P2 amino-purine transporter aided by the high affinity pentamidine transporter (HAPT 1) and the low affinity pentamidine transporter (LAPT 1) ((Mäser *et al*, 1999; de Koning, 2001) Similar to other diamidines, pentamidine has a predilection for DNA containing organelles, including the kinetoplast and nucleus, where it binds onto the minor groove of DNA at AT rich sites (Wilson *et al*., 2008). What happens thereafter is not known; it is however thought that the drug catalyses kinetoplast fragmentation, inhibition of glycolysis and/or interference with DNA dependent enzymes such as topoisomerase II RNA (Denise and Barrett, 2001; Tidwell and Boykin, 2003; Wilson *et al*., 2008).

Suramin

Suramin was first used against sleeping sickness in 1921 (WHO 2012), capping a successful medicinal chemistry effort in which more than 1000 naphthalene urea compounds were synthesized and evaluated for efficacy against trypanosomes (Steverding, 2010). Suramin is marketed as Germanin® (Bayer) in ampoules of 1 g and is effective against the first stage of both forms of HAT. However, suramin is clinically, used almost exclusively for *T. b. rhodesiense* infections since

pentamidine is considered to be a better choice for *T. b. gambiense* HAT. The most commonly used dosage regimen consists of a test dose of 4–5mg/kg body weight at day 1, followed by five injections of 20 mg/kg intravenously every 3–7 days (e.g. days 3, 5, 12, 19, 26) of suramin with a maximum dose per injection of 1 g (Burri, 2010). Suramin is extensively (99.7%) bound to plasma constituents including albumin and low density lipoproteins (Fairlamb and Bowman, 1980) (Denise and Barrett, 2001). The suramin-protein complex is taken up by trypanosomes via receptor mediated endocytosis (Denise and Barrett, 2001). Its mode of action is uncertain but is thought to include inhibition of enzymes including those responsible for respiration and glycolysis (Fairlamb and Bowman, 1980; Denise and Barrett, 2001). Pharmacokinetic studies revealed that suramin has a long half-life of 44–92 days (Burri, 2010). While suramin is better tolerated than the drugs used for late stage disease treatment, its use is associated with hypersensitivity reactions, albuminuria, haematuria and peripheral neuropathy (Burri, 2010). A bloodstream stage-specific invariant surface glycoprotein (ISG75) family mediates suramin uptake, and the AP1 adaptin complex, lysosomal proteases and major lysosomal transmembrane protein, as well as spermidine and N-acetylglucosamine biosynthesis, all contribute to suramin action (Alsford, 2012).

Melarsoprol

Melarsoprol, Arsobal® (sanofi-aventis), is a melaminophenyl based organic arsenical that was introduced as an anti-trypanosomal agent in 1949 (Pepin and Milord, 1994). It is accumulated into trypanosomes via P2, an aminopurine transporter that is encoded by the TbAT1 gene (Maser *et al.,* 1999). Deletions or mutations in the TbAT1 gene lead to drug resistance (Maser *et al.,* 1999) which may be one of the causes of high treatment failure rates that are sometimes reported from the field (Brun *et al*., 2001). Melarsoprol has for a long time been the first line treatment for late-stages of both *T. b. rhodesiense* and *T. b. gambiense* (WHO, 2012a). However the development of eflornithine in 1990 and the nifurtimox-eflornithine combination therapy (NECT) in 2009 (Priotto *et al*., 2009; WHO, 2012a), both better tolerated than melarspoprol, meant that clinicians now had better choices for management of West African sleeping sickness. Trypanosomes exposed to arsenicals lyse very rapidly, but the mode of action of the drug has also not been completely elucidated (Denise and Barrett, 2001). Melarsoprol has many undesirable side effects, the most significant being a post treatment reactive encephalopathy (PTRE) which occurs in 5-10% of treated patients and may be fatal in 50% of the affected patients (Kuzoe, 1993; WHO, 2012a).

Eflornithine

Eflornithine® (difluoromethylornithine [DFMO]), is the treatment of choice for second stage *T. b. gambiense* HAT, although it was first developed as an anti-cancer agent (WHO, 1998; Brun *et al.,* 2009). The uptake of eflornithine in *T. brucei* occurs via passive diffusion across the plasma membrane (Bitonti *et al.,* 1986). DFMO inhibits ornithine decarboxylase (ODC), which is involved in polyamine metabolism leading to cessation of trypanosomes growth. A functional immune system is required to kill the growth-arrested trypanosomes (Ghoda *et al.*, 1990). Its lack of activity against *T. b. rhodesiense* is thought to be due to higher overall activity and a shorter half-life of ODC in this subspecies compared to *T. b. gambiense* (Iten *et al.,* 1997).

Nifurtimox-Eflornithine Combination Therapy (NECT)

The Nifurtimox–Eflornithine Combination Therapy (NECT) has recently been included in the 16th WHO Essential Medicines list as treatment for 2nd stage sleeping sickness (Priotto *et al.,* 2009; WHO, 2012a). In NECT, eflornithine is administered intravenously at 200 mg/kg bid x 7 days, compared to 100 mg/kg four times per day x 14 days for monotherapy eflornithine (Priotto *et al*., 2009; Burri, 2010; WHO, 2012a) while nifurtimox is administered at 10mg/kg x 10 days per os. Thus, the key advantages of NECT are the simplification of eflornithine treatment, reduction of costs and overall reduction in hospitalization time for patients. In addition, NECT was shown to be equally effective to monotherapy eflornithine and to have comparable safety profiles (Priotto *et al*., 2009). However, NECT has not been evaluated against *T.b. rhodesiense* strains and is therefore not used for this indication.

New drugs for HAT

All the currently available HAT treatments have various shortcomings as highlighted above. The development of new drugs for HAT and other NTDs was however hardly being addressed in the period before year 2000 because these diseases do not offer good returns for the immense financial investments required to bring a drug to the market (www.thecpdd.org; Trouiller *et al*., 2002; Adams and Brantner, 2006; Chirac and Toreele, 2006). The gap in development of drugs for NTDs was well captured in Chirac and Toreele (2006) who demonstrated that out of 1556 drugs developed over the period from 1975 to 2004, only 21 (1.3%) were for NTDs and tuberculosis although these diseases accounted for 11.4% of the world's disease burden. Since the year 2000, public private partnerships (PPPs) which are also called product development partnerships (PDPs),

were formed to address the drug research and development (R&D) needs of NTDs with funding from public and philanthropic organizations and other donors (www.thecpdd.org. accessed July 24, 2012; Chatelain and Ioset, 2011). As a result the following compounds are now at different stages of the drug discovery and development process:

Fexinidazole

Fexinidazole is a nitroheterocyclic molecule that has recently shown promising activity against *T. brucei* strains (Kaiser *et al*, 2011) and visceral leishmaniasis (Wyllie *et al*., 2012). Fexinidazole was originally shown to have anti-trypanosomal activity by the drug company Hoechst in the 1980s but its development was, for unknown reasons, not pursued to clinical testing. It has only recently been rediscovered through compound mining and subsequent screening of at least 700 nitroheterocycles by the Drugs for Neglected Diseases initiative (DNDi) (Torreele *et al*., 2010). The in vitro activity of fexinidazole and its metabolites fexinidazole-sulfoxide and fexinidazole-sulfone are indicated by IC_{50} values ranging from 0.7 to 3.3 μM against both drug-sensitive and drug-resistant *T. brucei* spp (Kaiser *et al*., 2011. Fexinidazole was subsequently shown to cure *T. b. rhodesiense* and *T. b. gambiense* acute and chronic mouse models (Kaiser *et al*., 2011), was well tolerated in humans during phase I clinical trials (Torreele *et al*., 2010) and is scheduled to enter into phase II/III pivotal clinical trial in the third quarter of 2012 (http://www.dndi.org/portfolio/fexinidazole.html, accessed on 6th August 2012). Apart from an Ames positive result and the need for high dose rates to achieve in vivo efficacy, the available pharmacological data are all positive (Maser *et al*., 2012), justifying optimism that fexinidazole might complete the drug development programme successfully.

Benzoxaboroles

The oxaboroles are a promising new class of antimicrobials that contain boron in their structure. Screening an oxaborole library against *T. brucei* at Scynexis (Research Triangle Park, NC) and further activity based structural modifications led to the selection of SCYX-7158 as a clinical drug candidate for second stage HAT (Jacobs *et al*., 2011; Maser *et al*., 2012). The compound has an IC_{50} against *T.b. rhodesiense* and *T. b. gambiense* strains between 0.2-1 μM. SCYX-7158 also cures mouse models of both 1st and 2nd stage HAT (Jacobs *et al*., 2011). Phase 1 clinical trials were initiated in March 2012 by DNDi (Maser *et al.* 2012), ensuring that the HAT pipeline will not be empty even should an unexpected failure of fexinidazole occur.

Novel Diamidines

The Consortium for Parasitic Drug Development (CPDD) has focused on the diamidines class of compounds in their search for potential new therapeutic agents for neglected tropical diseases such as HAT. This was largely influenced by the success of pentamidine against *T. b. gambiense* since 1941 (WHO, 2012a). Continued interest in diamidines as antiparastic agents led the synthesis of novel aromatic diamidines by a group of researchers led by Dr David Boykin; six of these aromatic diamidines, including furamidine (DB75), were more active against *T. b. rhodesiense* than pentamidine (Das and Boykin, 1977). Despite their improved activity against trypanosomes, the new diamidines were, like pentamidine, not well absorbed after oral administration due to their positively charged amidine moieties. This was considered a significant limitation because of the need to develop easy to use oral drugs that would be more appropriate for the resource and infrastructure poor areas where HAT was endemic (Etchegory *et al*., 2001; Wilson *et al*., 2008).

In 1999/2000, the CPDD obtained funding from the Bill and Melinda Gates Foundation to develop furamidine (DB75) and its analogues for NTDs, including HAT. Since then, prodrugs were synthesised by masking the amidine moieties with alkoxy groups (Boykin *et al*., 1996; Ismail *et al*., 2003; Hua *et al*., 2009). The diamidine prodrugs were shown to be significantly more permeable across Caco-2 cell monolayers than their parent molecules (Ansede *et al*., 2005). In other studies, in vitro and in vivo (in mice) evaluation of prodrugs and/or active diamidines revealed that that these compounds had broad-spectrum activity against trypanosomes, *Leishmania* and *Plasmodium* species (Werbovetz, 2006), *Pneumocystis carinii (jirovecii)* pneumonia (Boykin *et al*., 1995, 1996; Hall *et al*., 1998; Tidwell and Boykin, 2003) and *T. cruzi* (Soeiro *et al.,* 2011). Furthermore, over 2000 diamidines were screened against a variety of *T. brucei* isolates in vitro, with 517 yielding an IC_{50} of less than 0.2μM and a selectivity of over 1000 against a mammalian cell line (L6) (Maser *et al*., 2012). These studies demonstrated the enormous potential of this class as source of potential new antiparasitic drugs.

This PhD study was anchored onto the diamidines development project with the objective of identifying molecules with suitable biological and pharmacological properties to be recommended for further development as potential HAT drugs.

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Chapter 2: Goals and Objectives

Goal

The goal of this thesis was to investigate the efficacy and pharmacology of selected novel diamidines in animal models in order to contribute to the development of safe, efficacious and easy to use treatments for both first and second stage human African trypanosomiasis (HAT).

Objectives

The study's specific objectives were:

1. To elucidate the oral bioavailability of prodrug DB289 and compare its efficacy with that of parenterally administered DB75 and pentamidine in different mouse models of first stage HAT.
2. To evaluate the efficacy of DB289 against first and second stage *T. b. rhodesiense* infections in vervet monkeys in order to understand its therapeutic range in primates.
3. To characterize the nature, target organs and determinants of DB844 toxicity in vervet monkeys and evaluate its pharmacokinetics and efficacy in the vervet monkey model of second stage HAT.
4. To determine the utility of prodrug DB868 as a potential therapeutic agent for first stage HAT through metabolism studies in liver microsomes, toxicity study in un-infected monkeys and pharmacokinetics and efficacy evaluation in the first stage monkey model of HAT.
5. To compare the efficacy and pharmacokinetics of an orally administered prodrug (DB868) with that of its intramuscularly administered active metabolite (DB829) against second stage *T. b. rhodesiense* infections in monkeys.

Chapter 3: Efficacy of the Pentamidine Analogue, DB75 and its Prodrug DB289, against Murine Models of African Sleeping Sickness

J.K. Thuita[1], S.M. Karanja[2], T. Wenzler[3], R.E. Mdachi[1], J.M. Ngotho[1], J.M. Kagira[1], D. Boykin[4], R. Tidwell[5] and R. Brun[3*]

[1]Trypanosomiasis Research Centre, Kenya Agricultural Research Institute (TRC-KARI) P.O Box 362, Kikuyu Kenya

[2] Jomo Kenyatta University of Agriculture and Technology (JKUAT), P.O. Box 62000, Nairobi Kenya

[3]Swiss Tropical and Public Health Institute, Socinstrasse 57, P.O Box, CH-4002 Basel, Switzerland.

[4]Georgia State University, Atlanta, GA 30303, USA

[5]University of North Carolina at Chapel Hill, North Carolina, USA

*Corresponding author, Reto Burn, Swiss Tropical Institute, P.O. Box, CH-4002 Basel, Switzerland. reto.brun@unibas.ch

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ABSTRACT

The choice of drugs for the treatment of sleeping sickness is extremely limited. To redress this situation, the recently synthesised diamidines, 2,5-bis(4-amidinophenyl)-furan (DB75, Furamidine) and its methamidoxime prodrug, 2,5-bis(4-amidinophenyl)-furan-bis-O-methylamidoxime (DB289, Pafuramidine) were, together with pentamidine, evaluated for efficacy in rodent models. The activity was compared in three common mouse models that mimic the first stage of human African sleeping sickness. The mice were infected with the pleormorphic *T. b. rhodesiense* strains KETRI2537 and STIB900 or with the monomorphic *T. b. brucei* strain STIB795. Importantly, DB75 showed enhanced activity over pentamidine at comparable doses in all three mouse models. Complete cures were achieved with oral dosing of the prodrug DB289 in all three models without any observed toxicity. This shows that the prodrug strategy was successful in terms of reducing toxicity and increasing efficacy and oral bioavailability. Thus, both DB75 and especially its orally active prodrug, DB289, provide a promising new treatment approach for early stage human African trypanosomiasis.

Key words: Furamidine, DB75, Pafuramidine, DB289, sleeping sickness, mouse models, African trypanosomiasis

INTRODUCTION

The number of drugs available for the treatment of sleeping sickness is extremely limited. Pentamidine isethionate (Pentacarinat®) and suramin (Moranyl®, Germanin®) are the only two drugs currently registered for the treatment of early stage disease that is characterised by localization of trypanosomes within the haemolymphatic system. Both drugs are highly effective against the West African form of the disease that is caused by *Trypanosoma brucei gambiense*. However, only suramin is recommended for the treatment of the East African form that is caused by *T. b. rhodesiense* since pentamidine is considered less effective (Apted, 1980). Similarly, two drugs, melarsoprol (MelB, Arsobal®) and eflornithine (Ornidyl®), are recommended for the treatment of the late (meningoencephalitic) stage of the *T. b. gambiense* disease, but only melarsoprol is effective against *T. b. rhodesiense* infections (WHO, 1998). This situation is compounded by the fact that all the current therapies are unsatisfactory for various reasons, including poor efficacy, unacceptable drug toxicity, upcoming resistance, costs and undesirable route of administration (Fairlamb, 2003).

Recently, renewed efforts in the development of new drugs for HAT and other pathogenic conditions, have led to the synthesis of an extensive collection of low toxicity pentamidine–like diamidines by an international consortium for parasitic drug development (CPDD) under the leadership of the University of North Carolina at Chapel Hill. The diamidine-appeal is based on their known broad spectrum of activity against protozoal and fungal pathogens, including African trypanosomes. Initially, these drugs were developed as treatment for the AIDS related *Pneumocystis carinii* pneumonia (PCP) and leishmaniasis (Tidwell *et al.,* 1990, Hall et al., 1998). Since the year 2000 studies on these compounds have been extended to cover HAT with the double aim of i) increasing the number of chemotherapeutic agents available for this neglected disease and ii) developing a product that is orally administrable and can therefore be used in the rural African settings where HAT is a major problem. In this study, the orally active prodrug, 2,5-bis(4-amidinophenyl)-furan-bis-O-methylamidoxime (Pafuramidine, DB289) was evaluated together with its active parent compound 2,5-bis(4-amidinophenyl)furan (Furamidine, DB75) and the related comparator, pentamidine. The compounds were evaluated for efficacy against early stage *T. brucei* (*T. b. rhodesiense* and *T. b. brucei*) infections, using established animal models at the Trypanosomiasis Research Centre of the Kenya Agricultural Research Institute (TRC-KARI) and at the Swiss Tropical Institute (STI).

MATERIALS AND METHODS

Mice

Adult female Swiss White mice were used in experiments for the KETRI 2537 model. The mice were obtained from colonies maintained at TRC-KARI. Adult female NMRI mice were used in experiments for the STIB900 and the STIB795 model at STI. The mice were obtained from RCC, Ittingen, Switzerland. They weighed between 20-30g at the beginning of the study and were house under standard conditions with pelleted food and water *ad libitum*.

Trypanosomes

T. b. rhodesiense KETRI2537 is a derivative of EATRO1989 (Fink and Schmidt, 1980). The isolate is stored as cryopreseved stabilates in the TRC cryobank.

T. b. brucei STIB795, a derivative of S427 was received from the International Laboratory for Research on Animal Diseases, Kenya, and was originally isolated from a *Glossina pallidipes* in Uganda in 1960. After passages in a sheep, a tsetse fly and several passages in mice, a clone was adapted to axenic cultivation *in vitro*. *T. b. rhodesiense* STIB900 is a derivative of STIB704. The pathogen was isolated from a male patient at St. Francis Hospital in Ifakara/Tanzania in 1982. After several passages in rodents and a cyclical passage in *Glossina morsitans morsitans*, a cloned population was adapted to axenic culture *in vitro*. STIB795 and STIB900 isolates are maintained in the STI trypanosome cryobank.

Trypanocidal Compounds

The two experimental compounds, 2,5-bis(4-amidinophenyl)-furan-bis-O-methylamidoxime) (DB289), and 2,5-bis(4-amidinophenyl)furan) (DB75), both yellow odourless powders, were obtained from Immtech International, USA through Professor Rick Tidwell of the University of North Carolina (UNC). Pentamidine isethionate, a white powder, was obtained from supplies donated by the WHO. At TRC, the compounds were reconstituted as per the manufacturer's instructions using distilled deionised water (DB75) and commercially available water of injection (pentamidine). DB289 stock suspension was prepared in a solvent consisting of deionised water (99.4%), Tween® 80 (0.1%), and carboxymethyl cellulose sodium (CMC, medium viscosity, 0.5%). All compounds were freshly prepared every two days, in concentrations ranging from 7.5 mg/ml to 0.078 mg/ml. At STI, all compounds were dissolved in DMSO and subsequently diluted with distilled sterile water to final maximal concentration of 10% DMSO for the STIB795 and the STIB900 models.

Experimental Design

KETRI2537 Mouse Model

Two sub-lethally irradiated (600rads, 5 minutes) donor Swiss White mice were each inoculated intraperitoneally with 0.2 ml of the thawed *T. b. rhodesiense* stabilates, diluted in phosphate saline glucose (PSG). When these mice had a circulating parasitaemia of antilog 7.8 to 8.1 as described by Herbert and Lumsden (1976), they were bled and used to infect the experimental group of Swiss White mice, each mouse receiving $1x10^4$ parasites in 0.2 ml of PSG. Immediately after infection, mice were randomly assigned to groups comprising six mice each. Parasitaemia was monitored daily using smears of tail-snip blood, a minimum of 20 fields being examined before a sample was designated negative.

Treatment was initiated on first detection of trypanosomes. Oral (per os, PO) treatments (DB289) were administered using a gavage needle for five consecutive days while intraperitoneal (IP) treatments (DB289, DB75 and pentamidine) were administered for four consecutive days. Intravenous (IV) DB75 was administered as a single injection only. Compound volume administered to the mice was maintained at 0.1ml /10g of mouse body weight (bwt). Six mice served as infected-untreated controls.

Post treatment parasitaemia was monitored thrice a week for 60 days. Monitoring for deaths was carried out daily. When mice relapsed, they were promptly euthanised by carbon dioxide inhalation and incinerated. Median time to relapse (MTR) was calculated in days post last treatment. All protocols and procedures used in the current study were reviewed and approved by the Trypanosomiasis Research Centre (TRC) Institutional Animal Care.

STIB795 and STIB900 Mouse Models

Four female NMRI mice were used per experimental group. Each mouse was inoculated intraperitoneally with $1x10^5$ bloodstream forms of STIB795 or STIB900 respectively. The cryopreserved stabilate containing 10% glycerol was suspended in PSG to obtain a trypanosome concentration of 4×10^5 /ml. Each mouse was injected with 0.25 ml. Compound treatment was initiated 3 days post infection on 4 consecutive days for all administration routes in a volume of 0.1ml /10g mouse. Parasitaemia was monitored using smears of tail-snip blood twice a week after treatment followed by once a week until 60 days post infection. Mice were considered cured when

there was no parasitaemia relapse detected in the tail blood over the 60 days observation period. Four mice served as infected-untreated controls. Mean survival days (MDS) were calculated in day of death post infection of parasitaemic mice. All protocols and procedures used in the current study were reviewed and approved by the local veterinary authorities of the Canton Basel-Stadt.

RESULTS

Disease Pattern KETRI2537

A pre-patent period of 3 (range = 3-5) days was observed. Peak parasitaemia was reached on day 8-9 post-infection. This first parasitaemia wave was well controlled, in some cases being reduced to below the detection limit of antilog 5.4 (Herbert and Lumsden, 1976). Subsequent waves were not as well controlled, with only slight fluctuations between peak and trough parasitaemia levels. At terminal disease stage the untreated control mice were euthanised when parasitaemia levels reached antilog 8.4. The mean survival times of the infected control animals was 33 (range = 24-47) days.

Disease Pattern STIB795 and STIB900

A prepatent period of 3 days was observed for STIB795 and one of 2.6 days (range = 2-3) for STIB900. Peak parasitaemia for both strains was reached on day 4 to 5 post-infection. The mice could not control parasitaemia and consequently died with a mean survival time of 6 (range = 5-8) days.

Efficacy against KETRI2537 Infections

In mice that were treated with DB289, clearance of parasites from the peripheral circulation began 48 hours after initiation of treatment and was complete in all groups six days after the first drug dose. DB289 when administered orally or IP at dose rates equal or greater than 4mg/kg resulted in 100% cure rates (Table 1). However, at 4x3.125 mg/kg bwt, IP administration resulted in a higher cure rate of 4/6 (67%, CI=22.3-95.7%) as compared to a cure rate of 2/6 (33%, CI=4.3-77.7%) for PO administration at 5x 3.125 mg/kg, but this difference was statistically not significant ($P>0.05$). DB289 was well tolerated with no toxicity detected at any of the dose levels tested, which was up to 5x75 mg/kg PO and 4x75 mg/kg IP.

DB75 achieved 100% cure rates at all IP dose regimes equal to or greater than 4x1.56 mg/kg bwt and at the IV dose regimes tested (25, and 12.5 mg/kg bwt, single dose). At the lowest dose tested, 4 x 0.78 mg/kg bwt IP a cure rate of 3/4 (75%) was obtained. DB75 was significantly toxic when used at 4x50 mg/kg bwt IP (5/6 deaths). However, the lowest dose at which significant toxicity was detected (4x50 mg/kg) was 32 times higher than the minimum curative dose (4x1.56 mg/kg), resulting in a reasonable therapeutic index. Pentamidine achieved 100% cure rate at 4x12.5 mg/kg bwt IP. The results at lower doses tested demonstrated a clear dose response with 80% cures at 4x6.25 mg/kg and 40% at 4x3.125 mg/kg bwt. No signs of toxicity could be observed at 4x12.5 mg/kg bwt.

Efficacy against STIB795 Infection

The data obtained with the *T. b. brucei* STIB795 model was comparable with the data of the *T. b. rhodesiense* KETRI2537 model.

DB289 administered PO resulted in a good dose response. The minimum curative dose was 4x10 mg/kg. At 4x5 mg/kg, 3/4 mice (75%) were cured. The total dose was equal to 5x4 mg/kg PO in the KETRI2537 infected mice, which was also the minimum curative dose. No cures were obtained at 4x2.5 mg/kg PO. However the survival was extended compared to the untreated control mice, indicating that DB289 still exhibited some activity at this low dose.

A good dose response with regard to cures was also obtained with IP treatment of DB75. A dose of 4x1.0 mg/kg resulted in cure of all animals while 4x0.5 mg/kg cured 3/4 mice (75%). The KETRI2537 data was comparable showing 3/4 cures at the similar dose of 4x0.78mg/kg IP. DB75 failed to cure at 4x0.25mg/kg but could prolong the survival time significantly to >32.5days compared to the untreated control mice, which survived only for 6 days post infection. Pentamidine gave complete cures at the lowest tested dose of 4x5 mg/kg IP.

Efficacy against STIB900 Infections

The *T. b. rhodesiense* STIB900 model is significantly more stringent as compared to STIB795 or KETRI2537. A 10-times higher PO administered dose of DB289 was needed to achieve complete cure as compared to the STIB795 or a 20-times higher dose as compared to KETRI2537. DB289 failed to give any cures at ≤4x10 mg/kg but it prolonged the mice survival significantly and in a dose dependent manner. The untreated control mice died on average on day 6. DB289 was also administered IP in the STIB900 model and 3/4 cures were obtained at 4x50 mg/kg.

At half the dose, all mice developed a parasitaemia relapse but the relapses were late and all 4 mice survived the 60 days observation period.

DB75 was not able to cure mice at doses up to 4x20 mg/kg bwt in the STIB900 mouse model. However, a clear dose response on the level of survival could be observed. The parasitaemia relapses occurred late particularly at 4x20 mg/kg IP indicating that this dose was close to the minimum curative dose. DB75 administration at 4x 20mg/kg IP was recently repeated and cure of single mice could be achieved (data not published).

IV administration of DB75 did not result in cures of infected mice. The survival of the treated mice was dose-dependent and comparable to the IP administration. The toxicity limit was lower with IV administration as compared to IP.

Pentamidine gave no cures up to the maximum dose of 4x25 mg/kg IP. The survival of the mice was in a similar range as after DB75 treatment but slightly shorter.

Table 1: Treatment of KETRI 2537 infections with DB289, DB75 and pentamidine

Drugs		
Dose: mg/kg	No cured/total	MST (range)
Pafuramidine (DB289, administered PO x 5 days)		
6.25	6/6	
4	6/6	
3.13	2/6	23.5 (16-32)
1.56	0/6	9.0 (6-13)
0.78	0/6	6.0 (4-9)
Pafuramidine (DB289, administered IP x 4 days)		
6.25	4/4	
4	6/6	
3.13	4/6	25 (19, 31)
1.56	1/6	16 (10-31)
0.78	0/6	12.0 (5-15)
Furamidine (DB75, administered IP x 4 days		
6.25	6/6	
3.13	3/3	
1.56	5/5	
0.78	3/4	21
Furamidine (DB75, administered IV x 1day		
25	6/6	
12.5	6/6	
Pentamidine (administered IP x 4 days)		
12.5	3/3	
6.25	4/5	10
3.13	2/5	14 (7, 21)
1.56	0/6	15 (5-15)
Controls	0/6	33 (24-47)

Key: Cure = mice survived for more than 60 days after last drug dose without showing a relapse
MTR= median time to relapse post last treatment or mean time to death post infection for control mice

Table 2: Treatment of STIB795 and STIB900 infections with DB289, DB75 and Pentamidine

Drugs	*T.b.brucei* **STIB 795**		*T.b.rhodesiense* **STIB 900**	
Dose: mg/kg	No cured/infected	MSD (range)	No cured/infected	MSD (range)
Furamidine (DB75, administered IP x 4 days				
4x 20	-		0/4	>52.5 (40->60)
4x 10	-		0/4	46.5 (38-55)
4x 5	-		0/4	35.5 (33-40)
4x 2.5	4/4		0/4	32.75 (31-35)
4x 1.0	4/4		-	
4x 0.5	3/4	>60	-	
4x 0.25	0/4	>32.5 (5, >60)	-	
Furamidine (DB75, administered IV x 4 days				
4x 15	-			toxic
4x 10	-		0/4	47.5 (32-58)
4x 5	-		0/4	33.75 (29-36)
4x 2.5	-		0/4	28.5 (24-34)
4x 1.25	-		0/4	25.75 (21-31)
Pafuramidine (DB289, administered IP x 4 days)				
4x 50	-		3/4	>60
4x 25	-		0/4	>60
Pafuramidine (DB289, administered PO x 4 days)				
4x 100	-	-	4/4	
4x 50	-	-	2/4	>60
4x 10	4/4		0/4	46.5 (31-55)
4x 5	3/4	6	0/4	30 (24-38)
4x 2.5	0/4	22 (5-53)	0/4	19.75 (14-26)
Pentamidine (administered IP x 4 days)				
4x 25	-	-	0/4	>60
4x 20	-	-	0/4	42.75 (39-47)
4x 10	4/4		0/4	28 (18-35)
4x 5	4/4		-	-
4x 2.5	4/4		-	
4x 1	3/4	> 60	-	
4x 0.5	3/4	58	-	
4x 0.25	0/4	>35 (24->60)	-	
control	0/4	6 (5-8)	0/4	6 (5-7)

Key: MSD: mean survival days post infection of the mice that got a parasitaemia relapse
Range: days of death post infection of the mice with parasitaemia
Cure= mice survived for more than 60days after infection without showing a parasitaemia relapse
Control: infected mice without any drug treatment

DISCUSSION

Pentamidine and the diamidine DB75 and its prodrug DB289 showed excellent activities in the *T. b. rhodesiense* KETRI2537 mouse model of infection. This trypanosome strain is routinely used in the vervet monkey model at TRC (Schmidt and Sayer, 1982). Importantly, 100% cure rates were achieved at dose rates of 4x12.5 mg/kg bwt IP with pentamidine, 4x4 mg/kg bwt IP and 5x4 mg/kg bwt PO with DB289 and 4x1.56 mg/kg bwt IP with DB75. Comparable results were obtained using the *T. b. brucei* STIB795 mouse model. 100% cure rates were achieved at dose rates of ≤ 4x5 mg/kg bwt IP with pentamidine (testing of lower doses ongoing), 4x 10 mg/kg PO with DB289 and 4x1 mg/kg bwt IP with DB75. The *T .b. rhodesiense* STIB900 mouse model is very stringent and hard to cure as compared to STIB795 although both strains show similar in vitro drug sensitivity (Bernhard, 2006). High level of parasitaemia was reached more rapidly with STIB900 and clearance after treatment with DB75 took more time compared to STIB795 (Bernhard, 2006). Pentamidine could not cure the STIB900 model, not even at the highest dose tested (4x25 mg/kg IP). Also DB75, which cured easily the KETRI2537 and STIB795 model, did not give any cures in the STIB900 model. However, DB75 prolonged the mice's survival more than pentamidine at identical dosages. A higher dose of DB75 was not used as a preliminary toxicity test did show toxicity at 1x100 mg/kg IP. DB289, the prodrug of DB75, achieved complete cures in the STIB900 mouse model without showing any toxicity at 4x100 mg/kg PO. Partial cure (50%) was achieved at 4x50 mg/kg PO. Intraperitoneal administration of DB289 resulted in insignificantly higher activity. Approximately 10-times more prodrug was needed to achieve comparable cure rates in the STIB900 model as compared to KETRI2537 and STIB795.

Interestingly, DB289 showed good oral efficacy indicating that sufficient quantities were absorbed from the mouse gastrointestinal tract, confirming that the prodrug approach is an effective way of delivering dicationic drugs across the gut mucosa (Tidwell *et al*., 1990; Hall *et al*., 1998). Oral efficacy presents the exciting new possibility to treat HAT patients without the need for hospitalisation, thus making the treatment of a neglected tropical disease that affects the world's poorest people easier to manage and cheaper (WHO, 1998; Etchegorry *et al*., 2001). Oral diamidine activity has earlier been reported for diminazene aceturate (Berenil®) in a substantial number of HAT patients (Abaru *et al*., 1984) although this drug is not registered for human use.

All the drugs were well tolerated, except intravenous administered DB75 had toxic effects at 4x15 mg/kg or intraperitoneally at the high dose of 4 x 50 mg/kg bwt. No adverse effects were

noticed for the prodrug DB289 at dose levels up to 5 x 75 mg/kg bwt in the KETRI2537 model, a dose that is 18.8 times higher than the minimum curative dose. Also at 4 x 100 mg/kg bwt which cured the highly stringent STIB900 model no signs of toxicity could be observed. This compares well with pentamidine and diminazene aceturate which, when administered in mice at dose rates as high as 50 mg/kg did not cause overt signs of toxicity (Zweygarth and Rottcher, 1989). Thus, although susceptibilities of *T. b. rhodesiense* clinical isolates to drugs are known to vary widely (Bacchi et al., 1990, 1994 and 1997), the wide safety margin suggests that there is adequate room for adjustment of dose rates to cater for such differences between isolates.

In conclusion, this study has shown that DB75 is much more potent than pentamidine and that the prodrug DB289 cures the *T. b. rhodesiense* mouse models when administered PO. This supports the expectation that DB289, which is currently in phase III clinical trials, may successfully become the first oral treatment of human African trypanosomiasis.

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Chapter 4: Efficacy of the Novel Diamidine Compound 2,5-Bis(4-amidinophenyl)-furan-bis-O-Methlylamidoxime (Pafuramidine, DB289) against *Trypanosoma brucei rhodesiense* Infection in Vervet Monkeys after Oral Administration

R. E. Mdachi[1],* J. K. Thuita[1] J. M. Kagira[1] J. M. Ngotho[1,4], G. A. Murilla[1] J. M. Ndung'u,[1,5] R. R. Tidwell,[2] J. E. Hall,[2] and R. Brun[3]

[1]Kenya Agricultural Research Institute-Trypanosomiasis Research Institute, P.O. Box 362, 00902 Kikuyu, Kenya1;

[2]University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-75252; [3]Swiss Tropical and Public Health Institute, CH-4002 Basel, Switzerland;

[4]Institute of Primate Research, Nairobi, Kenya;

[5]Foundation for Innovative New Diagnostics, 71 Av. Louis-Casaï,

Case Postale 93, 1216 Cointrin, Geneva, Switzerland

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ABSTRACT

Owing to the lack of oral drugs for human African trypanosomiasis, patients have to be hospitalized for 10 to 30 days to facilitate treatment with parenterally administered medicines. The efficacy of a novel orally administered prodrug, 2,5-bis(4-amidinophenyl)-furan-bis-O-methlylamidoxime (pafuramidine, DB289), was tested in the vervet monkey (*Chlorocebus* [*Cercopithecus*] *aethiops*) model of sleeping sickness. Five groups of three animals each were infected intravenously with 10^4 *Trypanosoma brucei rhodesiense* KETRI 2537 cells. On the seventh day postinfection (p.i.) in an early-stage infection, animals in groups 1, 2, and 3 were treated orally with pafuramidine at dose rates of 1, 3, or 10 mg/kg of body weight, respectively, for five consecutive days. The animals in groups 4 and 5 were treated with 10 mg/kg for 10 consecutive days starting on the 14th day p.i. (group 4) or on the 28th day p.i. (group 5), when these animals were in the late stage of the disease. In the groups treated in the early stage, 10 mg/kg of pafuramidine completely cured all three monkeys, whereas lower doses of 3 mg/kg and 1 mg/kg cured only one of three and zero of three monkeys, respectively. Treatment of late-stage infections resulted in cure rates of one of three (group 4) and zero of three (group 5) monkeys. These studies demonstrated that pafuramidine was orally active in monkeys with early-stage *T. brucei rhodesiense* infections at dose rates above 3 mg/kg for 5 days. It was also evident that the drug attained only minimal efficacy against late-stage infections, indicating the limited ability of the molecule to cross the blood-brain barrier. This study has shown that oral diamidines have potential for the treatment of early-stage sleeping sickness.

INTRODUCTION

Human African trypanosomiasis (HAT) (sleeping sickness) is caused by the flagellated protozoan parasites Trypanosoma brucei gambiense and T. brucei rhodesiense and is inevitably fatal when untreated. The disease is endemic to sub-Saharan Africa, where an estimated 50 million people in more than 20 countries are considered to be at risk (23). WHO statistics show that from 1998 to 2004, the number of HAT cases declined from 37,991 to 17,616 (30), and the estimated prevalence declined in the same period, from 300,000 to 500,000 cases to 50,000 to 70, 000, even though the number of people covered by active screening almost doubled in the same period (30). These declining trends are indicators that the partnerships between individual African governments and the WHO are having a positive impact on the control of HAT. However, these gains could easily be lost through political instability and/or the outbreak of wars, which inevitably lead to a breakdown in surveillance activities (10). At present, HAT remains a major health problem in certain foci in the Democratic Republic of Congo, Angola, and Southern Sudan (10, 30). Since the trypanosomes' remarkable ability to vary their major surface antigen makes successful vaccine development unlikely in the near future (26), the only recourse available is to intensify research and the development of new drugs.

Current drugs that are used to treat HAT include pentamidine, suramin, melarsoprol, and eflornithine (5). These are far from ideal, since they elicit substantial toxic effects and/or require parenteral application, which makes them difficult to administer in the rural African settings where the disease typically occurs. Furthermore, melarsoprol, the only drug available that is effective against the late stages of both *T. brucei gambiense* and *T. brucei rhodesiense* disease, has shown incidences of treatment failure of >25% in epidemic areas of northern Uganda and northern Angola (4, 7, 8). Both melarsoprol and eflornithine require long periods of hospitalization and monitoring owing to the complicated treatment schedules needed (1). In addition, melarsoprol may cause reactive encephalopathy; this occurs in 5 to 10% of treated patients and has a fatality rate of ~50% (5). The provision of an oral drug of low toxicity for sleeping sickness would allow an effective administration of the required doses with minimal supervision from medical personnel. This is an important consideration, since enough qualified people are rarely available in those parts of Africa where the disease is endemic.

A recently synthesized novel prodrug, 2,5-bis(4-amidinophenyl)-furan-bis-O-methlylamidoxime (pafuramidine, DB289) exhibited excellent oral activity and reduced acute toxicity in acute and chronic mouse models of trypanosomiasis (3, 12, 27). The enhanced oral activity of DB289 is attributed to an improved oral absorption of the prodrug in which the cationic

functionalities are masked and to its conversion to the active compound 2,5-bis(4-amidinophenyl)furan (furamidine; DB75) in the liver. DB75 strongly binds to the minor groove of DNA at AT-rich sites and weakly binds by intercalation at the GC sites of DNA (25). Consequently, its subsequent mode of action involves interference with the normal function of the pathogen's DNA-dependent enzymes, perhaps topoisomerase II (2). In spite of pafuramidine being an analogue of pentamidine, it appears to possess two properties that would overcome the two disadvantages of pentamidine, namely, parenteral administration and lack of efficacy against central nervous system (CNS) infection.

Recently, following prolonged administration with the drug in a clinical trial for AIDS related *Pneumocystis cariini* pneumonia, abnormal liver enzyme values were found in several volunteers. However, no subject required any treatment or hospitalization for this abnormality. Despite this, pafuramidine is evidently a promising candidate for use as a new drug for oral administration. It was therefore tested in the vervet monkey model of sleeping sickness at the Trypanosomiasis Research Centre of the Kenya Agricultural Research Institute. This nonhuman primate model is known to mimic the human disease closely (21) and is a valuable tool for preclinical chemotherapeutic studies. Experiments were conducted to determine the efficacy of DB289 after multiple oral doses against both early- and late-stage infections with *T. brucei rhodesiense*.

MATERIALS AND METHODS

Experimental Animals

Fifteen vervet monkeys (*Chrolocebus* [*Cercopithecus*] *aethiops*) of both sexes and weighing between 2.5 and 4.5 kg were obtained from the Institute of Primate Research in Kenya. They were housed in quarantine for a minimum of 90 days and screened for evidence of disease, including zoonotic infections. They were also dewormed using albendazole (Valbazen; Utravetis Ltd., Kenya) and treated for ectoparasites using carbaryl (1-naphthyl methylcarbamate) (Sevin; Bayer Ltd., Kenya). During that period, the monkeys became accustomed to staying in individual squeeze-back stainless steel cages and to being handled. They were fed twice daily on fresh vegetables and commercial monkey cubes (Unga Ltd., Kenya) and given water ad libitum. Before the study, the animals were transferred to experimental wards and acclimatized for a further 2 weeks.

Trypanosomes

The *T. brucei rhodesiense* stabilate used was KETRI 2537, a derivative of EATRO 1989, isolated from a patient in Uganda by direct inoculation of blood and lymph node aspirates into a monkey and later cryopreserved (9).

Test Drug.

Pafuramidine (DB289) [2,5-bis(4-amidinophenyl)-furan-bis-O-methlylamidoxime; MediChem, Lemont, IL] was provided by Immtech Pharmaceuticals, Inc., as a yellow powder.

Drug Preparation

DB289 was prepared as a 1% suspension in distilled water containing 0.5% (wt/vol) carboxymethylcellulose sodium and 0.1% (wt/vol) Tween 80. The preparation was stored at 4°C and used for up to 3 days before a fresh preparation was made.

Drug Administration

The DB289 suspension was administered orally using a gavage needle once daily for 5 or 10 days depending on the treatment regimen for each group.

Experimental Design

The 15 monkeys were randomized into five treatment groups without sex bias (Tables 1 and 2). The monkeys were all infected by intravenous injection of 10^4 trypanosomes diluted from the infected blood of immunosuppressed donor mice using phosphate-buffered saline as previously described for the vervet monkey model (11, 17). On the seventh day postinfection (p.i.) in an early-stage infection, animals in groups 1, 2, and 3 were treated orally with pafuramidine at dose rates of 1, 3, and 10 mg/kg, respectively, for five consecutive days. The animals in the remaining two groups, groups 4 and 5, were treated with 10 mg/kg for 10 consecutive days, starting on the 14th day p.i. (group 4) or on the 28th day p.i. (group 5). The monkeys were monitored for clinical changes and for the presence of trypanosomes in body fluids. Once parasites were detected in any of the body fluids, the monkeys were removed from the study through humane euthanasia.

Table 1: Body weight and PCV changes in vervet monkeys infected with *T. brucei rhodesiense* and treated with different regimens of pafuramidine

Group (dose [mg/kg])	Mean preinfection wt (kg) ± SE	% Wt change at [a]			Mean preinfection % PCV ± SE	% Change in PCV at [a]		
		Day 0 p.t. [b]	Day 28p.t.[c]	End point		Day 0 p.t. [b]	Day 28 p.t.[c]	End point
1 (1)	3.15 ± 0.45	−2.20	−2.1 [d]	0	47.0 ± 4.4	−4.30	−17.0 [d]	−23.40
2 (3)	3.18 ± 0.33	−5.00	−6.60	−1.60	46.0 ± 4.4	−9.40	−2.90	−3.60
3 (10)	2.88 ± 0.43	−6.30	0	+3.50	40.3 ± 2.5	−5.80	−1.60	+13.20
4 (10)	4.25 ± 0.52	−4.20	−5.20	−0.70	51.7 ± 6.1	−25.80	−13.50	−7.70
5 (10)	3.32 ± 0.60	−10.50	−8.10	−2.10	47.7 ± 1.5	−41.30	−9.10	+1.40

[a] = Percent change in body weight or PCV from preinfection values, – indicates a decline, + indicates an increase; [b] = Values taken before the first treatment dose, i.e., on the 7th day p.i. for groups 1 to 3, 14th day p.i. for group 4, and 28th day p.i. for group 5; [c] = Data for groups 2 to 5 at 28 days p.t.; [d] = Percent change in body weight and PCV at 1 day p.t. for group 1.

Table 2: Outcomes of treatment of *T. brucei rhodesiense*-infected vervet monkeys with different regimens of pafuramidine (DB289)

Group	Dose (mg/kg) (length of treatment [days])	Monkey	Time to detection of trypanosomes in biological fluids (days p.t.)				No. of cured monkeys/no. of total monkeys
			Blood		CSF		
			MIT	WS + HCT	MIT	HC + CT	
1	1 (5)	463	7	9	NC	NC	0/3
		484	NC	4	7	14	
		485		3	14	21	
2	3 (5)	482	55	79		21	1/3
		483	69	66	69	55	
		411	Cured				
3	10 (5)	454	Cured				3/3
		468	Cured				
		486	Cured				
4	10 (10)	471	56	87	NC	NC	1/3
		478	Cured				
		488	ND		42	ND	
5	10 (10)	490	ND		21	42	0/3
		501	56	73	NC		
		520	ND		119	119	

ND, not detected for >180 days p.t.; WS, wet smear; HC, hemocytometer counting; CT, concentration techniques for CSF; NC, trypanosome not cleared.

Monitoring and Sample Collection

Physical examinations and sampling of body fluids were carried out weekly for the first 28 days and then once every 2 weeks up to 100 days post treatment and monthly thereafter until the end of the experiment. Physical examinations and collection of blood and cerebrospinal fluid (CSF) were undertaken on monkeys anesthetized with diazepam (May and Baker, United Kingdom) at 1 mg/kg body weight and with ketamine hydrochloride (Rotexmedica, Trittau, Germany) at 15 mg/kg body weight. Post treatment monitoring was maintained for a period of 180 days.

At each time point, approximately 1 ml of blood was collected into EDTA by inguinal venipuncture. In addition to blood samples, 1.5 ml of CSF was collected by lumbar puncture of the anesthetized monkeys (11). Blood collected by inguinal venipuncture was used for determinations of the packed cell volume (PCV) using the microhematocrit method. White cells in the CSF were counted using a hemocytometer chamber.

Determination of Parasitaemia

The determination of parasitaemia started 3 days p.i. It was done by examination of wet films of ear prick blood according to a method described previously by Herbert and Lumsden (13) (detection limit, 105 trypanosomes/ml of blood) or by the hematocrit centrifugation technique (HCT) according to methods described previously by Woo (31) (detection limit, 103 trypanosomes/ml of blood). Blood collected by inguinal venipuncture was used for parasitemia determinations using both the wet-film and HCT methods.

For estimation of parasites in the CSF, some of the free-flowing CSF was collected into a capillary tube and immediately transferred into a hemocytometer chamber to count the number of trypanosomes and white cells. Negative CSF samples were then centrifuged and reexamined for trypanosomes. In cases where no parasites were detected in either blood or CSF by the above-described methods, Swiss white mice were used to test for the presence of trypanosomes (11). Samples of 0.2 ml of blood or CSF were inoculated intraperitoneally into two mice per sample.

RESULTS

Clinical Disease before Treatment

Following infection, trypanosomes were detected in wet smears of peripheral blood with a mean period of 4.2 days (range, 3 to 5 days). Enlargement of peripheral axillar and inguinal lymph nodes was observed by the seventh day p.i., and enlargement of the spleen was observed by the 14th day p.i. The monkeys became dull, had rough hair coats, and showed a transient loss of appetite. The rectal body temperature rose from a preinfection (0 days p.i.) value of 38.2°C ± 0.2°C to 38.9°C ± 0.2°C (mean ± standard error). The rise in temperature was significant ($P = 0.01$) and coincided with peak parasitemia levels of 7×10^7 trypanosomes/ml of blood. Other clinical signs included loss of body weight as the infection progressed. The decrease in mean body weight was marginal (<6% of preinfection weight) for animals treated while in the first stage of disease at 7 to 11 days p.i. (groups 1 to 3), and a more substantial change (10.5%) was found for animals (group 5) treated during late-stage disease at 28 days p.i. (Table 1). The changes in mean PCV were similarly

marginal for animals treated while in the early stage but more pronounced for those treated while in the late stage of disease (Table 1). Animals that were treated in the late stage of disease also had confirmed trypanosomes in CSF (1 to 6 trypanosomes/µl) and elevated white cell counts (mean, 10 cells/µl of CSF; range, 4 to 20 cells/µl of CSF).

Results of Treatment with Pafuramidine

Pafuramidine treatment was shown to benefit most of the monkeys. Body weights and PCV improved in all monkeys by the time of termination of the experiment except for those in group 1 (Table 1). In some individuals, there was clearance of both parasitemia and CSF parasitosis (Table 2). However, the onset and durability of the beneficial effects of pafuramidine treatment varied in the different groups.

Group 1 (1 mg/kg for 5 days per os)

In group 1, trypanosomes became undetectable in the blood by direct microscopy or HCT a day after the last (fifth) drug dose and remained so for a mean of 5 days (range, 3 to 9 days). However, even during this period of apparent aparasitemia, viable trypanosomes were isolated from the monkeys' blood by the mouse inoculation test (MIT), indicating that blood parasites were never completely eliminated. In the CSF, trypanosomes were detected first by MIT on 7 to 14 days posttreatment (p.t.) or 14 to 21 days p.t. by microscopy (direct hemocytometer counting or concentration techniques), indicating that the drug treatment delayed the onset of trypanosomes in the CSF. Clinical data, especially the PCV, continued to decline (Table 1), indicating that at the low dose of 1 mg/kg of body weight, pafuramidine treatment did not cure any of the monkey infections. The monkeys were humanely euthanized.

Group 2 (3 mg/kg for 5 days per os)

Animals in group 2 became parasitologically negative by both blood and CSF on the fifth day of dosing. However, two of the three monkeys experienced recrudescence of trypanosomes, first in the CSF and later in the blood (Table 2). In these two animals, CSF trypanosomes were first detected by the MIT at 21 days p.t. and then by direct microscopy (Table 2). In this group, treatment led to improvement in both mean weight and mean PCV (Table 1) although only one of three the monkeys fulfilled the criteria of cure at 180 days p.t. The two monkeys that relapsed were humanely euthanized.

Group 3 (10 mg/kg for 5 days per os)

Animals in group 3 became parasitologically negative by the fifth day of dosing and remained so until the end of the experimental period. Similarly, no trypanosomes were recovered by MIT throughout the 180 days of p.t. monitoring. Meanwhile, weight and PCV returned to preinfection levels by 28 days p.t. (Table 1), and all clinical aberrations resolved, underlining that all three of three monkeys were cured of the infection.

Group 4 (10 mg/kg for 10 days per os starting 14 days p.i.)

In group 4, following treatment, both weight and PCV improved, although not to preinfection levels (Table 1). All three monkeys in this group became aparasitemic by day 5 of dosing, but only two of three monkeys (monkeys 478 and 488) remained free of blood parasites until 180 days p.t. In the CSF, either trypanosomes were not cleared (monkey 471) or recrudescence occurred within the first 42 days p.t. (monkey 488) (Table 2). Only monkey 478 remained free of trypanosomes in both blood and CSF as determined by HCT and MIT and was considered to be cured (Table 2). The results were confirmed by the pattern of white cell changes in the CSF; these oscillated between 0 and 12 cells/µl of CSF for monkey 478, which was cured, but occasional peaks of above 25 cells/µl of CSF, which declined to lower peaks below 15 cells/µl of CSF, were observed in monkey 488, where there was recrudescence of the infection (Fig.1).

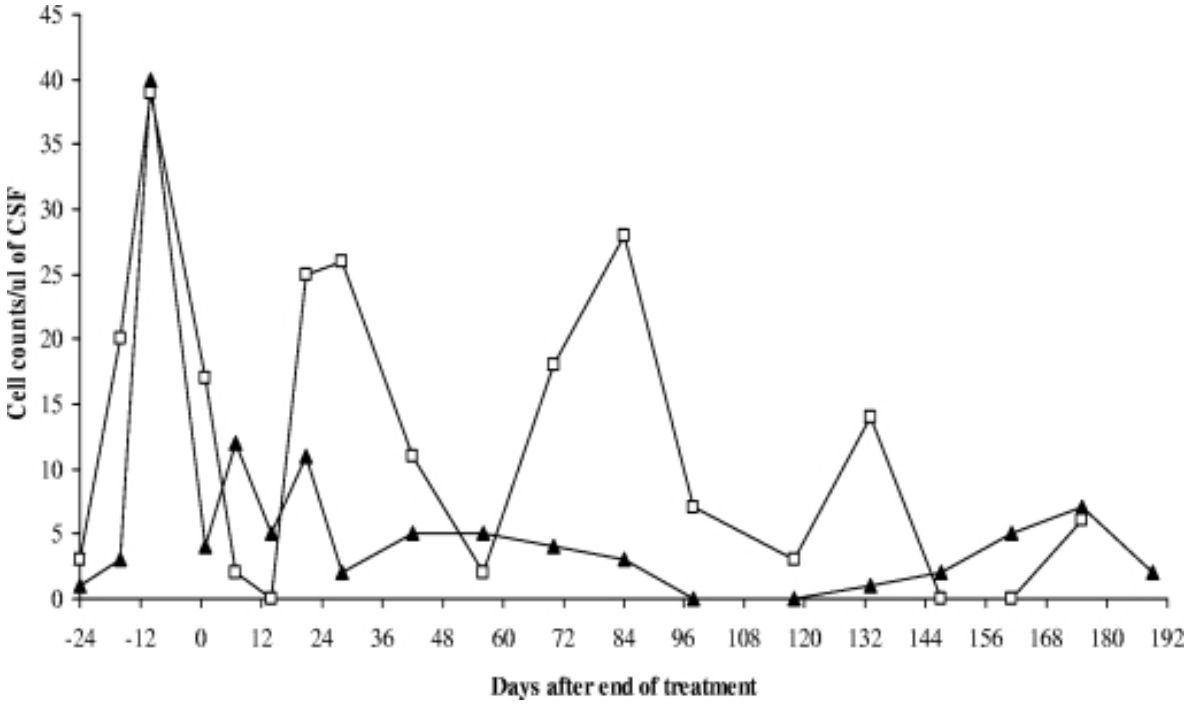

FIG. 1: CSF white cell changes in vervet monkeys 478 (▲) and 488 (□) after treatment with pafuramidine at 10 mg/kg for 10 days while in the late stage (14 days p.i.) of disease. Note that in vervet monkey 478, levels of white cells declined after treatment and remained low throughout the observation period, while in vervet monkey 488, the drop in white cell number was transient and showed recrudescences to >25 cells/µl of CSF

Group 5 (10 mg/kg for 10 days per os starting at 28 days p.i.)

In group 5, all the animals became aparasitemic by day 4 of dosing. However, only two of three of them remained free of blood trypanosomes by the end of the 180 days of p.t. monitoring (Table 2). In the CSF, trypanosomes were not cleared in monkey 501, while in the remaining two monkeys, the trypanosomes disappeared after treatment, and recrudescence occurred (Table 2). The significant improvement in weight and PCV (Table 1) was probably due to the absence of trypanosomes from the hemolymphatic system in spite of the presence of trypanosomes in the CNS.

DISCUSSION

In this study, the oral administration of pafuramidine (DB289) had a profound effect on established *T. brucei rhodesiense* infections in vervet monkeys. In the acute disease stage, when the monkeys showed high parasitemia levels, treatment with 10 mg/kg orally for five consecutive days cleared three of the three monkeys in group 3 of their trypanosome infections and kept them trypanosome free for the entire monitoring period of 180 days. In addition, the treatment reversed the clinical symptoms of trypanosomiasis, thus fulfilling all criteria for confirmation of cure.

Comparison of this group of monkeys with groups 1 and 2, which also had an early-stage infection with a 5-day treatment schedule (days 7 to 11), shows a clear dose response to pafuramidine. At 3 mg/kg, only one of three animals was cured, and at the lowest dose, 1 mg/kg, all animals relapsed within 1 week after treatment or did not clear parasitemia at all.

The clinical signs described in this report for acute infection mirror those described in previous reports for syringe passage of *T. brucei rhodesiense* infections in vervet monkeys (15, 21), tsetse-transmitted infections in vervet monkeys (28), and early-stage infections in sleeping sickness patients (16, 18, 22). These clinical manifestations are evidence of hemolymphatic system involvement. The fact that the clinical manifestations were reversed when pafuramidine was administered in sufficient concentrations indicates that the drug was able to access and eliminate trypanosomes from all hemolymphatic sites.

In the two groups of monkeys with a late-stage infection, treatment with 10 mg/kg for 10 days cured only one of three monkeys treated from 14 days p.i. (group 4) and none in the group treated from 28 days p.i. onwards. In group 4, the presence of trypanosomes and white cell counts above the 5-cell/mm3 cutoff for confirmation of late-stage disease (29) was demonstrated in the CSF of the infected monkeys. The fact that trypanosomes were eliminated from the CSF in one animal and the mean PCV in the group was improved (from −25.8% to −13.5%) (Table 1) suggests that orally administered pafuramidine has some ability to cross the blood-brain barrier. Although the levels of pafuramidine (DB289) and its active form, furamidine (DB75), in CSF were not determined in the present study, it is likely that the variation in individual responses resulted from variation in the CSF levels of the drugs. Generally, the activity of a drug may show considerable interanimal variation, which results in different concentrations at the site of action (kinetic differences) or different responses to a given drug concentration (dynamic differences) (19). Studies with melarsoprol, the drug used to treat late-stage sleeping sickness, similarly indicated that the peak levels attained in the CSF of monkeys were about 50 times less than peak levels in plasma

and were insufficient in some animals to eliminate all trypanosomes from the CSF compartment (6).

The fact that none of the animals treated with pafuramidine at 10 mg/kg for 10 days starting at 28 days p.i. was cured shows that as the residence time of trypanosomes in the CSF increased, the infections became more difficult to treat. It has been postulated that the invasion of the CSF by trypanosomes progressively leads to parasite invasion of the perivascular spaces (Virchow-Robin spaces) and from there to the parenchyma of the CNS (20). Further evidence of the importance of the residence time of trypanosomes in CSF was provided previously by Schmidt and Sayer (21), who found that one monkey with a disease duration of 107 days had histologically demonstrable meningoencephalitis. Although our study had no component of histology and could therefore not confirm or rule out the presence of meningoencephalitis at 28 days p.i., it is clear that the levels of the active drug (DB75) attained in the CSF were inadequate to eliminate trypanosomes from the sites to which they were sequestered, making the CNS the main source of relapse parasites, as previously observed for mice (14). Even in this group of monkeys, however, the drug cleared bloodstream-form trypanosomes and maintained two of three monkeys trypanosome free throughout the monitoring period, which provides further evidence of the ability of pafuramidine to access hemolymphatic but not CNS sites of the infected monkeys.

To conclude, the study showed that the novel prodrug pafuramidine (DB289) was effective against early *T. brucei rhodesiense* infection in monkeys. In addition, the drug was well tolerated by all the infected monkeys, confirming previous observations of uninfected vervet monkeys (John Thuita, TRC-KARI, personal communication). The drug was generally not effective against late-stage disease and may therefore not be indicated for this use. However, Sturk et al. (24) previously observed that after intravenous administration in mice, there was a fivefold increase in brain levels of pafuramidine with parenchymal localization of compound fluorescence, which suggested that the unaltered prodrug does penetrate the blood-brain barrier and may be subject to in situ biotransformation. Since the present study showed that pafuramidine was efficacious in clearing trypanosomes from the bloodstream, it would be worth considering the testing of parenteral treatment regimens to improve the bioavailability of the drug in the CNS in nonhuman primates. However, the issue of recently found human toxicity should be taken in consideration in further nonhuman primate studies.

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Chapter 5: Pharmacology of DB844, an Orally Active aza Analogue of Pafuramidine, in a Monkey Model of Second Stage Human African Trypanosomiasis

*[1]John K. Thuita, [2]Michael Z. Wang, [1]John M. Kagira, [3]Cathrine L. Denton, [3]Mary F. Paine, [1]Raymond E. Mdachi, [1]Grace A. Murilla, [4]Shelley Ching, [5]David W. Boykin, [6]Richard R. Tidwell, [6]James E. Hall and *[7]Reto Brun.

[1]Trypanosomiasis Research Centre, Kenya Agricultural Research Institute (TRC-KARI) P.O box 362-00902, Kikuyu, Kenya.
[2] Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas, U.S.A.
[3] Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, North Carolina, U.S.A.
[4]SVC Associates, Inc., Apex, North Carolina, U.S.A
[5] Chemistry Department, Georgia State University, Atlanta, Georgia, U.S.A.
[6] Pathology Department, School of Medicine, University of North Carolina at Chapel Hill, North Carolina, U.S.A.
[7]Swiss Tropical and Public Health Institute and University of Basel, Socinstrasse 57, CH-4002 Basel, Switzerland
*Corresponding authors' e-mail addresses: thuitajk@yahoo.com; reto.brun@unibas.ch

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ABSTRACT

Novel drugs to treat human African trypanosomiasis (HAT) are still urgently needed despite the recent addition of nifurtimox-eflornithine combination therapy (NECT) to WHO Model Lists of Essential Medicines against second stage HAT, where parasites have invaded the central nervous system (CNS). The pharmacology of a potential orally available lead compound, N-methoxy-6-{5-[4-(N-methoxyamidino) phenyl]-furan-2-yl}-nicotinamidine (DB844), was evaluated in a vervet monkey model of second stage HAT, following promising results in mice. DB844 was administered orally to vervet monkeys, beginning 28 days post infection (DPI) with *Trypanosoma brucei rhodesiense* KETRI 2537. DB844 was absorbed and converted to the active metabolite 6-[5-(4-phenylamidinophenyl)-furanyl-2-yl]-nicotinamide (DB820), exhibiting plasma C_{max} values of 430 and 190 nM for DB844 and DB820, respectively, after the 14^{th} dose at 6 mg/kg qd. A 100-fold reduction in blood trypanosome counts was observed within 24 h of the third dose and, at the end of treatment evaluation performed four days post the last drug dose, trypanosomes were not detected in the blood or cerebrospinal fluid of any monkey. However, some animals relapsed during the 300 days of post treatment monitoring, resulting in a cure rate of 3/8 (37.5%) and 3/7 (42.9%) for the 5 mg/kg x 10 days and the 6 mg/kg x 14 days dose regimens respectively. These DB844 efficacy data were an improvement compared with pentamidine and pafuramidine both of which were previously shown to be non-curative in this model of CNS stage HAT. These data show that synthesis of novel diamidines with improved activity against CNS-stage HAT was possible.

AUTHOR SUMMARY

New drugs are needed to treat sleeping sickness, especially the second stage of the disease, which is characterised by the presence of parasites (trypanosomes) in the brain. The purpose of this work was to determine whether DB844, a new drug that is converted to the active form (DB820) after oral administration, has the potential to treat second stage sleeping sickness. Two dosing regimens of DB844 were evaluated in two groups of vervet monkeys that were infected experimentally with trypanosomes. Treatment was initiated four weeks after infection, when the monkeys were in second stage sleeping sickness, as confirmed by the presence of trypanosomes in brain fluid. Orally administered DB844 was well absorbed, tolerated and resulted in a decrease of trypanosomes in both the blood and brain fluid. However, some monkeys relapsed after treatment, with an overall cure rate of approximately 40% in both study groups. For at least two days after last dosing, the active drug, DB820, achieved blood concentrations known to be at least 19 times more than the minimum concentration that has been shown to be effective against a stringent human infective trypanosome isolate (STIB 900). These results represent an advance in efforts to develop new related compounds as oral treatments for sleeping sickness.

INTRODUCTION

Human African trypanosomiasis (HAT, sleeping sickness) is a debilitating disease that is caused by the protozoan parasites, *Trypanosoma brucei gambiense* and *T. b. rhodesiense.* The disease is transmitted by tsetse flies (*Glossina spp*) and is therefore endemic only in geographical areas (foci) where both the parasite and vector are present; these foci are distributed in ~20 sub-Saharan African countries and are home to at least 50 million people who are at risk of contracting HAT [1]. The spatial and temporal distribution of HAT is further determined by emergence of virulent parasite strains, breakdown in control and/or surveillance activities, changes in climate and vegetation and movements of carrier livestock species across borders [2]. As a result, the epidemiology of HAT is characterised by periodic epidemics interspersed with periods of near total eradication [3]. In 2009, the annual incidence dropped below 10,000 reported cases for the first time in 50 years, a success credited to the World Health Organization (WHO), national disease control programmes, bilateral co-operation and non-governmental organizations [4]. However, control activities must be maintained and new diagnostics and drugs developed to have a realistic chance of eventually eliminating HAT, a disease which has a history of reversing previous gains [2, 5-7].

Modern drug research and development activities for HAT have recently increased markedly, primarily through the efforts of public private partnerships (PPP's), which are funded by governmental and philanthropic organizations. A first success of these PPP's is the nifurtimox-eflornithine combination therapy (NECT), a product that has was recently added to the WHO essential medicines list for the management of *T. b. gambiense* CNS-stage infections [4, 8]. In addition, the dimethoxyamidine prodrug pafuramidine (DB289), a pentamidine-like compound developed by the Consortium for Parasitic Drug Development (CPDD), became the first oral drug to enter phase III clinical trials for 1st stage HAT [9]. Clinical and preclinical investigations on pafuramidine (DB289) demonstrated that oral diamidine prodrugs could achieve efficacy equal to or better than pentamidine in the management of 1st stage HAT [9-12]. However, the development program was terminated after some subjects (6%) in an extended phase I clinical trial (14 day dose regimen of 100 mg bid) developed delayed renal insufficiency [9]. Like pentamidine, pafuramidine did not achieve cure against 2nd stage HAT in animal models [11, 12].

In an effort to develop a compound that was well tolerated and with efficacy against 2nd stage HAT, a next-in-class dimethoxyamidine prodrug, N-methoxy-6-{5-[4-(N-methoxyamidino)

phenyl]-furan-2-yl}-nicotinamidine (DB844), was evaluated. Similar to the biotransformation of DB289, DB844 was shown to be sequentially *O*-demethylated and *N*-dehydroxylated in human liver microsomes to form its active metabolite, 6-[5-(4-phenylamidinophenyl)-furanyl-2-yl]-nicotinamide (DB820) [13]. In mice, oral DB844 appeared to be well absorbed and converted to DB820, and cured all animals (5/5) in the GVR35 CNS model of HAT [11]. In vitro, DB820 was a potent trypanocide with an IC_{50} value of 2.4 ng/ml (5.2 nM) against *T. b. rhodesiense* STIB900 [11]. DB820 also accumulated in the DNA containing organelles and bound to DNA molecules preferentially at AT rich sites, thus likely sharing the same mechanism of action with pentamidine [14-16]. The purpose of this study was to further evaluate the potential of DB844 as a novel oral treatment against 2nd stage HAT by characterizing its pharmacology in vervet monkeys, a species commonly used as a preclinical model for HAT [12]. The specific study objectives were to (a) characterize the metabolic profile of the prodrug in vervet monkey liver microsomes; (b) evaluate the toxicity of orally administered DB844 in un-infected monkeys to understand tolerability and to define an appropriate dose range for efficacy studies in the monkey HAT model; and (c) evaluate the pharmacokinetics, efficacy and safety of DB844 in the infected vervet monkey model, which closely mimics human sleeping sickness.

MATERIALS AND METHODS

Ethics Statement

Studies were undertaken in adherence to experimental guidelines and procedures approved by the Institutional Animal Care and Use Committee (IACUC), the ethical review committee for the use of laboratory animals at the Trypanosomiasis Research Centre of the Kenya Agricultural Research Institute (TRC-KARI). The experimental guidelines also complied with National guidelines of the Kenya Veterinary Association.

Trypanocidal Test Compound

The test compound N-methoxy-6-{5-[4-(N-methoxyamidino) phenyl]-furan-2-yl}-nicotinamidine (DB844) (Figure 1) was synthesized in the laboratory of Dr. David Boykin (Georgia State University, Atlanta, GA, USA) as previously reported [17]. The current study used DB844 (lot D, $C_{19}H_{19}N_5O_3 \bullet 3HCl \bullet H_2O$) with a purity of > 95% as determined by both NMR [17] and high performance liquid chromatography (HPLC)/UV (described below). DB844 was supplied to KARI-TRC through CPDD in the form of yellow powder in opaque and water tight bottles. Once received, the drug containing bottle was wrapped in aluminium foil and refrigerated at 4-8°C. Dosing formulations were prepared daily by dissolving the drug in de-ionised distilled water to render concentrations of 5 and 6 mg/ml. Reconstituted drug was protected from light by wrapping drug-containing vials with aluminium foil.

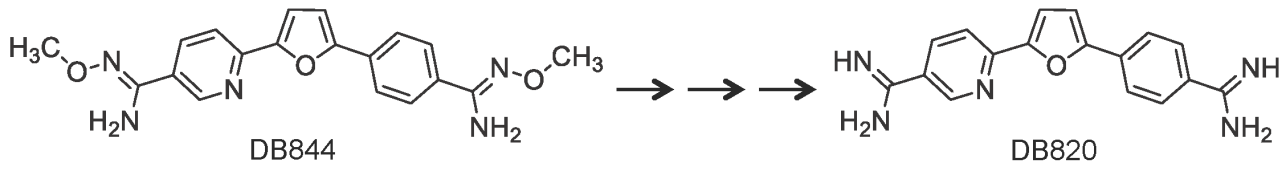

Figure 1: Chemical structure of DB844 and DB820, doi:10.1371/journal.pntd.0001734.g001

Trypanosome Isolate

A pleomorphic isolate, *T.b rhodesiense* KETRI 2537, a derivative of EATRO 1989 that was isolated from a patient in Uganda by direct inoculation of blood and lymph node aspirate into a monkey and later cryopreserved at KARI- TRC [18], was used. This isolate is the basis of the KETRI vervet monkey and mouse models and has been widely used for drug efficacy trials [19, 20].

Experimental Animals

Adult vervet monkeys [*Chlorocebus (Cercopithecus) aethiops* syn. African green monkeys] (n = 22) weighing between 2.5 and 5.5 kg were acquired from the Institute of Primate Research (IPR) in Kenya. Monkeys were quarantined, screened, acclimated for the study for a minimum of 90 days while being screened for evidence of disease as previously described [12, 20, 21]. They were also de-wormed, treated for ectoparasites, and acclimated to staying in individual squeeze-back stainless steel cages and human handling. The monkeys were fed a diet of fresh vegetables and commercial monkey cubes (Unga feeds, Nakuru Kenya) twice daily and provided water *ad libitum.*

Metabolism of DB844 in Vervet Monkey Liver Microsomes

The metabolism of DB844 was investigated using liver microsomes, prepared from a male vervet monkey by XenoTech, LLC (Lenexa, KS), in the presence of NADPH as described previously [22] with modifications. Briefly, incubation mixtures (1 ml at pH7.4, in triplicate) contained 10 μM DB844 and 0.2 mg/ml monkey liver microsomes. After a 5-min equilibration period at 37°C, reactions were initiated with the addition of NADPH. Aliquots (50 μl) of the reaction mixtures were removed at 0, 5, 10, 15, 30, 60, 90, and 120 min and mixed with 25 μl of ice-cold acetonitrile. The mixtures were centrifuged (10,000 x g for 5 min at 4°C) and the supernatants were analyzed by HPLC/UV using the same method as previously described for DB289 [22]. Metabolite identification was performed by comparing retention times to those of synthetic standards, which include M1A (DB1284), M1B (DB1058), M2A (DB1285), M2B (DB1212), M3 (DB821), and DB820 [13]. DB844 and metabolites were quantified using a single-concentration calibration curve generated using synthetic standards.

Toxicity Study in Uninfected Monkeys

The toxicity of DB844 was evaluated in uninfected vervet monkeys with the aim of defining the appropriate dose-range for the compound, identifying target organs of toxicity and characterizing the nature of drug-induced toxicity in this species. Six monkeys were used to evaluate the tolerability of 10-day oral dose regimens. Baseline clinical and haematology data were collected during a 14-day period, after which two monkeys (one male and one female) per dose group were administered with DB844 at 5, 10 or 20 mg/kg/day for 10 days via oral gavage. A dose volume of 1 ml/kg was administered. Daily ward rounds were conducted to assess feed intake (appetite), demeanour, posture and stool composition and consistency. Feed intake was assessed by

scoring the proportion of the daily ration consumed by each monkey based on a scale of 1 (full ration eaten), 3/4, 1/2, 1/4 and 0 (no feed eaten) as previously described [21].

Monkeys were monitored for 28 days post dosing. They were anaesthetized through intramuscular (IM) injection with ketamine HCl (10-15 mg/kg) and Valium® (0.5 mg/kg) to facilitate physical examination, body weight measurements and sample collection. Blood was collected from the femoral vein *via* inguinal venipuncture as described previously [20, 23] and divided into aliquots: 1 ml in EDTA for full haemogram determination and 2 ml in EDTA for plasma separation. Plasma was separated using a cool spin centrifuge (4°C, 1500 revolutions/minute), separated into aliquots and stored at -20°C pending analysis for DB844/DB820 concentrations.

When overt drug related toxicity was detected, drug administration was withdrawn from the affected monkey(s) to allow the affected individuals to recover. Monkeys that failed to recover were humanely euthanized using 20% pentobarbitone sodium (Euthatal®, Rhone Merieux) for gross and histopathology examination. Euthanasia was carried out when monkeys were judged to have deteriorated to the *in extremis* condition, characterised by inability or reluctance to perch and very low feed intake (less than 1/4 of daily ration) for 2-3 consecutive days [21, 24]. Organ specimens from these monkeys were preserved in 10% formalin and later sectioned for histopathogy. The processed slides were stained with haematoxylin and eosin.

Monkey Infections

Sixteen vervet monkeys in two groups of eight monkeys each (four males and four females) were used. After a 14-day baseline weight, clinical and haematology data collection period, the sixteen monkeys were infected by intravenous injection of approximately 10^4 trypanosomes diluted from infected blood of immuno-suppressed donor Swiss white mice [12]. Parasitaemia post infection was determined by examination of wet film of ear prick blood and/or examination of buffy coat after centrifugation of blood collected in a heparinised capillary tube as described previously [25]. Parasitaemia in wet film was estimated using the rapid matching method of Herbert and Lumsden [26]. In addition, monkeys were confirmed to be in second stage disease by detection of trypanosomes in the CSF with or without elevated white cell counts above 5 cells/µl [4, 20, 21, 23]. At 28 days post infection (DPI), the animals were treated with DB844 via oral gavage at 5 mg/kg qd x 10 days (group I, n = 8) or 6 mg/kg qd x 14 days (group II, n = 8), utilising a dose volume of 1 ml/kg. The monkeys were examined for parasitaemia every day during drug treatment

and then twice weekly until ≥ 300 days post dosing, at which point monkeys were considered cured if they remained clinically normal and parasite-free as determined below.

Pre and Post Treatment Monitoring

Daily ward rounds were conducted throughout the study to assess feed intake, demeanour, posture, and stool composition and consistency. Feed intake was assessed by scoring the proportion of the daily ration consumed by each monkey on a scale of 1 (full ration eaten), 3/4, 1/2, ¼, and 0 (no feed eaten) as previously described [21]. Monkeys were anaesthetised weekly by intramuscular injection of ketamine HCl (10-15 mg/kg) and Valium® (0.5 mg/kg) for physical examination, body weight measurements and collection of whole blood in EDTA and cerebrospinal fluid (CSF) samples. Blood samples (1 ml) were thereafter collected for preparation of plasma for pharmacokinetic studies at 1, 2, 4, 8, 24, 48, 96 and 168 h and then weekly until 28 days, while CSF samples (0.7-1.5 ml) were collected at 1, 24, 96, 168 h and then weekly until 28 days post last dosing. Samples were collected by inguinal venipuncture (blood) or lumbar puncture (CSF) of anaesthetised monkeys as previously described [12]. Plasma was separated using a cool spin centrifuge (4°C, 1500 RPM). After 28 days post last dosing, blood and CSF samples were collected once every two weeks up to 100 days, then once per month until 300 days post dosing for haematology and parasite detection only. During sampling, some of the free-flowing CSF was collected into a capillary tube and immediately transferred onto a haemocytometer (Neubaeur) chamber for counting of trypanosomes and/or white blood cells. Samples that were negative for CSF trypanosomes by direct microscopy were concentrated and examined according to the modified single centrifugation technique [21, 27]. All the CSF samples that remained negative for trypanosomes after the concentration step were then sub-inoculated into Swiss white mice (2 mice per sample) to further aid in diagnosis of infected fluids. Similarly, blood samples that were negative for trypanosomes after concentration [26] were inoculated into Swiss white mice. When trypanosomes were detected in blood and/or CSF or when monkeys were diagnosed to have attained *in extremis* condition as previously described [21, 24], they were humanely euthanized using 20% pentobarbitone sodium (Euthatal®, Rhone Merieux) for post mortem examination.

Haematology samples (1 ml) were analysed using an $AC^{3diff}T$ Coulter Counter (Miami, Florida, USA). Clinical chemistries were determined using a Humalyzer analyser system. Finally, plasma and CSF were analyzed for drug and metabolite concentrations using an HPLC-tandem Mass Spectrometry (HPLC-MS/MS) procedure as described below.

Sample Preparation and HPLC-MS/MS Quantification

Monkey plasma and CSF samples were prepared and quantified for DB844 and DB820 using previously described methods [22, 28] with modifications. Briefly, plasma or CSF samples (25 µl) were extracted with 200 µl of 7:1 (v/v) methanol:water containing 0.1% (v/v) trifluoroacetic acid and deuterated internal standards (30 nM each for DB844-d4 and DB820-d4), followed by centrifugation, evaporation, and reconstitution before HPLC-MS/MS analysis [28]. HPLC-MS/MS quantification of DB844 and DB820 was performed on an Applied Biosystems (Foster City, CA) API 4000 triple quadruple mass spectrometer equipped with a Turbo IonSpray interface in positive ion mode (MDS Sciex, San Francisco, CA). Reconstituted samples (4-5 µl) were separated on an Aquasil C18 analytical column 2.1 × 50 mm, 5 µm (Thermo Electron, Waltham, MA) with mobile phases consisting of HPLC-grade water containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B). After a 0.4-min initial hold at 15% B, mobile phase composition began with 15% B and was increased to 80% B over 1.6 min, followed by a 1.0-min hold, at a flow rate of 0.5 ml/min. The column was then washed with 95% B for 1.3 min at a flow rate of 0.5 ml/min and was re-equilibrated with 15% B at a flow rate of 0.5 ml/min for 0.5 min before injection of the next sample. The characteristic SRM transitions for DB844 and DB820 were m/z 366.2 → 319.2 and 306.2 → 289.2, respectively. The calibration curves for DB844 ranged from 2.5 – 2500 nM and 1 – 1000 nM in plasma and CSF, respectively, using a quadratic equation with 1/x weighting. The calibration curves for DB820 ranged from 10 – 2500 nM and 1 – 1000 nM in plasma and CSF, respectively, using a quadratic equation with 1/x weighting.

Data Analysis

Data were analysed statistically using Statview for Windows Version 5.0.1 (SAS Institute Inc, Cary, NC). Repeated measures ANOVA, with Fishers PLSD post hoc test, was used to test the effects of trypanosomal infection, as well as DB844, on haematology and clinical chemistry parameters in comparison with respective baseline values ($\alpha = 0.05$). Confidence intervals [95%] were derived to further test the significance of observed findings. Pharmacokinetic outcomes were determined using standard non-compartmental methods performed using Phoenix WinNonlin (version 6.2, Pharsight, Mountain View, CA).

RESULTS

Metabolism Profiles in Monkey Liver Microsomes

DB844 was rapidly metabolized in vervet monkey liver microsomes (MLM) with a microsomal half-life of approximately 14 min to form at least seven metabolites over a 120 min incubation period (Figure 2). The first two metabolites to be detected, M1A and M1B, were likely formed through the oxidative *O*-demethylation of either the pyridyl or phenyl side of DB844 [13]. M1A and M1B gave rise to M2A and M2B respectively, through reductive *N*-dehydroxylation, or further O-demethylation to form the bis-amidoxime metabolite, M3. The *O*-demethylation of M2A and M2B resulted in M4A and M4B, respectively, which could also be generated by *N*-dehydroxylation of M3. At last, the *N*-dehydroxylation of M4A and M4B gave rise to the active metabolite DB820 (Figure 2). Metabolites M1A and M1B attained the highest concentrations during the initial 20 minutes of incubation after which M3 became the metabolite with the highest concentration in the drug/liver microsome mixture (Figure 2).

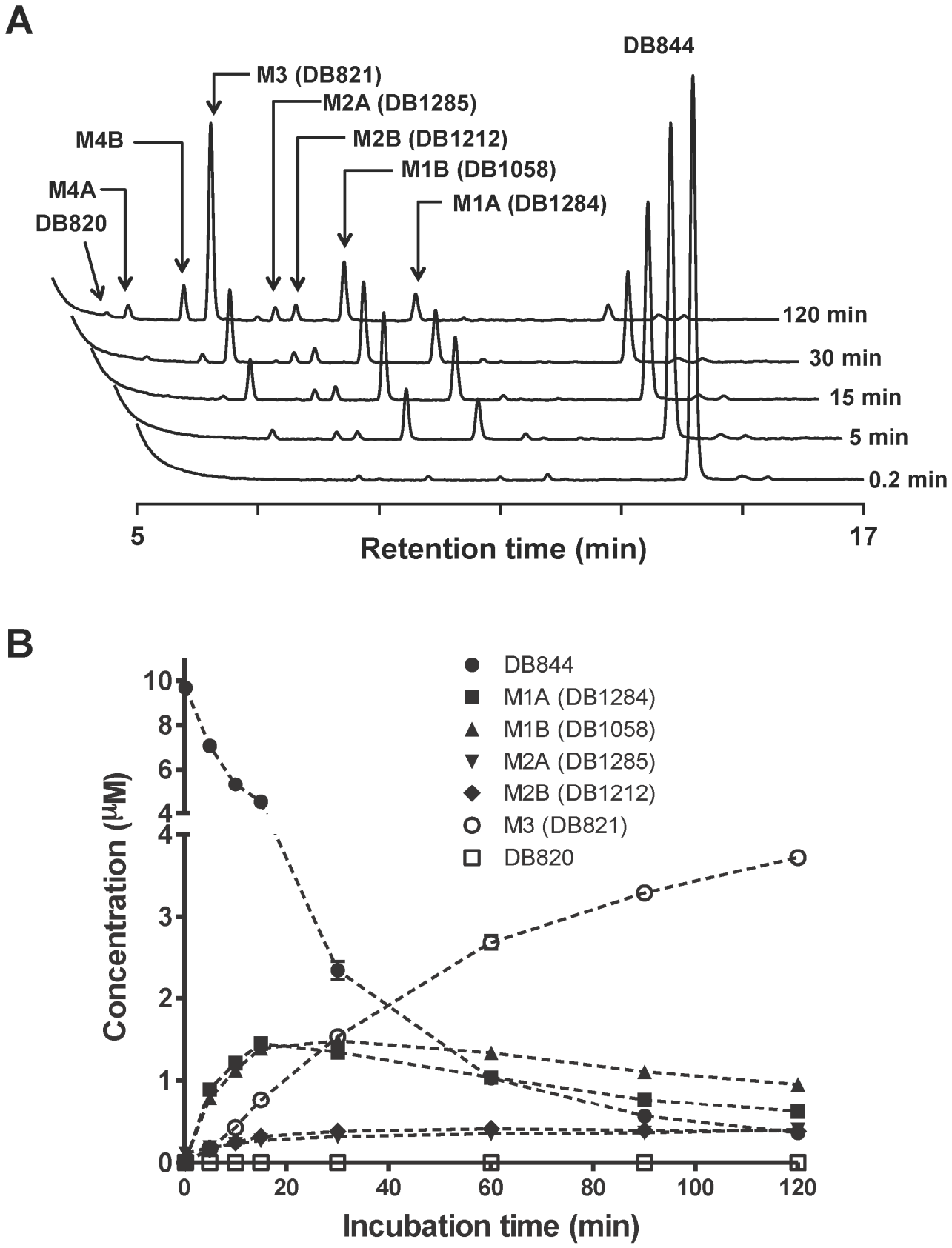

Figure 2: HPLC/UV chromatograms and concentration-time profiles of DB844/metabolites

following incubation of DB844 with male vervet monkey liver microsomes. A: HPLC/UV chromatograms; B: Concentration-time profiles of DB844 and metabolites. Incubation mixtures (1 ml at pH7.4, in triplicate) contained 10 μM DB844 and 0.2 mg/ml monkey liver microsomes. Aliquots were taken at 0.2, 5, 15, 30, and 120 min and evaluated for concentrations of DB844 and six metabolites (M1A, M1B, M2A, M2B, M3, and DB820). Metabolites M4A and M4B were not quantified due to lack of synthetic standards

Toxicity

Uninfected monkeys were orally dosed with DB844 at 5, 10 or 20 mg/kg/day for 10 days. The lowest, 5 mg/kg, did not elicit adverse clinical signs of toxicity in 2/2 monkeys (Table 1). Overt toxicity was however observed in 1/2 and 2/2 monkeys to which DB844 was administered at 10 and 20 mg/kg, respectively (Table 1). In the high dose group, overt toxicity was confirmed, at the earliest, after eight daily doses (cumulative dose [CD] = 160 mg/kg). Drug administration to these monkeys was immediately withdrawn to allow clinical recovery which, however, did not occur. As a result, the monkeys were humanely euthanized 1-2 days later (9-10 days post first dosing) (Table 1). Both monkeys from the middle (10 mg/kg) dose group completed the 10-day dose regimen successfully (CD = 100 mg/kg) after which 1/2 developed signs of overt toxicity and was eventually euthanized 16 days post first dosing (Table 1). The adverse clinical events included anorexia, gastrointestinal disturbances (vomiting or changes in stool consistency), jaundice and weight loss of up to 10.5% (Table 1). Haematology revealed nothing significant except for vervet 578 (10 mg/kg); in this monkey, the red cell distribution width (RDW) rose from 15 to 18.4 (23%) while mean corpuscular volume (MCV) rose from 76.6 to 86.9fl (13%). At histopathology examination, lesions observed included inflammation and erosions of the gastrointestinal tract (GIT), fatty change (steatosis) in the liver, hydropic degeneration of renal tubular cells and haemorrhage and haemosiderosis in multiple organs (Table 1). Toxicokinetic analysis revealed that in the two monkeys that were dosed at 5 mg/kg, DB844 and DB820 achieved average concentrations of 215 nM and 41.6 nM respectively at 1 h post last dosing. However, other toxicokinetic measurements could not be determined due to the limited sampling.

Table 1: Adverse events in monkeys treated orally with DB844.

Parameters evaluated		I: DB 844 at 5 mg/kg x 10 days orally		II: DB844 at 10 mg/kg x 10 days orally		III: DB844 at 20 mg/kg x 10 days orally	
		572F	582M	541F	578M	543F	606M
Adverse clinical events	Reduced feed intake	None	None	Yes (11)	Yes (14)	Yes (8)	Yes (9)
	GIT changes	None	None	None	Yes (16)	Yes (8)	Yes (8)
	Jaundice	None	None	None	yes	yes	yes
	% weight loss	1.8	4.9	4.6	10.5	9.1	6.5
	Daily doses completed	10/10	10/10	10/10	10/10	8/10	8/10
	Euthanised due to toxicity	No	No	No	Yes (16)	Yes (9)	Yes (10)
Liver histology	Fatty change (Steatosis)	NA	NA	NA	+++	+++	+++
	Inflammation	NA	NA	NA	++	++	+++
	Focal necrosis	NA	NA	NA	+++	+++	+++
	Haemosiderosis	NA	NA	NA	++	+	+++
GIT histology	Ulcers/erosions	NA	NA	NA	+++	+++	+++
	Inflammation	NA	NA	NA	+++	+++	+++
	Haemosiderosis	NA	NA	NA	++	+	+++
Spleen histology	Expanded red pulp	NA	NA	NA	+++	++	++
	Haemosiderosis	NA	NA	NA	+++	+++	+++
Kidney histology	Hydropic degeneration/ interstitial oedema	NA	NA	NA	+++	+	+

Key: GIT = gastrointestinal system; numbers in parenthesis = time in days post first drug dose when an adverse clinical event was observed; NA = not assessed since the monkeys were not euthanized; F = female; M = male

Progression of the *T.b. rhodesiense* Infection

The pre-patent period of the experimental *T. b. rhodesiense* infection in both groups of monkeys was approximately 5-6 days (Table 2). Parasitaemia rose to a peak of 5.0 x 10^7 trypanosomes (antilog 7.7) within 2-3 days (7-8 DPI) but subsequently fluctuated to give characteristic waves of parasitaemia (Figure 3). Monkeys developed a classical *T. b. rhodesiense* clinical disease characterised by reduction in feed intake, raised hair coats, reduced activity, dullness and/or excitability when the clinical signs were first observed at 4-7 DPI. Anorexia and inactivity were transient, lasting a maximum of 3 days before normal appetite and activity were regained. Enlargement of peripheral lymph nodes (especially axillar and inguinal lymph nodes) and splenomegaly (up to 3 times compared to pre-infection) were also observed 7-14 DPI while facial, scrotal or eyelid oedema were observed in 8/16 (50%) of the infected monkeys from 20 DPI. Average (± SE) weight before infection was 3.6 ± 0.4 (range = 2.6-5.5) and 3.1 ± 0.3 (range = 2.3-4.2) kg for group I and II, respectively. Four weeks after infection, weight decreased significantly ($p = 0.0003$) by 6.4% and 5.6%, respectively. Time to parasitization of the cerebrospinal fluid (CSF) was a median 21 days (range = 7-27) for both groups of monkeys (Table 2). At 27 DPI, one day before initiation of treatment with DB844, trypanosome numbers ranged from 1-8/µl of CSF; median cell numbers were 5.0 (range = 0-45) and 6.0 (range =1-20) cells/µl of CSF in groups I and II respectively. During previous weekly samplings, white cell counts in some monkeys increased to 152/µl of CSF (Table 2).

Efficacy

At 28 DPI when monkeys had shown characteristic features of 2nd stage infection (i.e., presence of trypanosomes and elevated white blood cell counts above 5/µl of CSF), they were treated with DB844 at 5 mg/kg x 10 days (group I) or 6 mg/kg x 14 days (group II). At 24 h post third drug dose (i.e., 4th day of treatment), trypanosomes were not detected in wet smears of peripheral blood (Figure 3), showing that at least 100-fold reduction in parasitaemia had been achieved, from 10^7 to 10^5 trypanosomes/ml of blood which is the detection limit of the matching method of Herbert and Lumsden [23]. End of treatment (EoT) evaluation was conducted at 1 and 4 days post last dosing time points. At the one day post dosing time point, trypanosomes were detected in some monkeys using sensitive trypanosome concentration techniques for both blood and CSF [25, 27] as evidenced by provisional cure rates of 7/8 (group I) and 5/7 (group II) (Table 2). The three monkeys with persisting low numbers of trypanosomes in the blood and/or CSF

eventually tested negative at the 4 days post dosing time point (Table 2), demonstrating an EoT provisional cure rate of 100% for both groups.

Post treatment follow-up was carried out for at least 300 days post dosing. During this follow-up period, nine monkeys relapsed demonstrating an overall test of cure rate of 3/8 (37.5%) and 3/7 (42.5%) for the 5 mg/kg and 6 mg/kg dose groups respectively (Table 2). Trypanosomes were observed exclusively in the CSF in five of the relapsed monkeys. In three of the four remaining relapse cases, trypanosomes were detected in the CSF earlier than in the blood. Overall, the median (range) time to trypanosome recrudescence was 133 (35-322, n = 9) days for CSF and 261 (239-322, n =3) days for blood trypanosomes. Despite trypanosomes becoming cleared from the peripheral blood by eighth day of dosing, monkey 625 (Table 2) developed toxicity and was humanely euthanized 2 days after administration of 10^{th} dose of DB844 at 6 mg/kg (CD = 60 mg/kg). At post mortem examination, liver and gastrointestinal toxicity were observed, comparable to findings in 1/2 (10 mg/kg) and 2/2 (20 mg/kg) un-infected monkeys euthanized due to DB844 toxicity.

Haematology Changes in the Infected Monkey Model

The trypanosome infection provoked a reduction in erythrocytes (red blood cells, RBC) and associated parameters. Average haemoglobin concentration declined by 32.1% in group II monkeys, from 13.4 ± 0.6 [95% CI = 12.4-15.1] g/dl at baseline (day 0) to 9.1 ± 0.6 [95% CI =7.9-10.3] g/dl ($p < 0.0001$] at 27 DPI (Table 3). Erythrocyte counts and haematocrit concentration, declined significantly ($p < 0.0001$, Table 3) by 28.7% and 32.1%, respectfully. Mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) decreased significantly ($p < 0.0001$) (Table 3). Erythrocyte associated parameters of group I monkeys exhibited similar trends (data not shown), indicating that the infection caused a microcytic hypochromic type of anaemia. Monkeys also experienced significant thrombocytopenia and leucopaenia (Table 3) related to the experimental *T. b. rhodesiense* infection. Upon treatment with oral DB844, no drug related haematology changes were observed. Trypanosome induced anaemia, thrombocytopenia and leucopaenia resolved rapidly. Baseline white cells numbers were re-established by end of treatment while platelet and RBC associated parameters were re-established within seven and 28-63 days respectively (Table 3; Figure 4).

Table 2: Treatment outcome in monkeys treated with DB844 while in second stage *T. b. rhodesiense* infection

Group	Monkey ID	PP (DPI)	Time to CSF parasitization (DPI)	Tryps/µl CSF at 27 DPI	WC/µl of CSF at 27 DPI	EoT test at 1 day post last dose	EoT test at 4 days post last dose	TOC test at 300 days post last dose
I	566	5	7	1	4 [17]	Neg	Neg	Cured
	568	5	27	1	45 [45]	Neg	Neg	Cured
	576	5	21	1	2 [12]	Neg	Neg	Relapsed
	599	6	14	8	39 [152]	Pos	Neg	Relapsed
	601	6	21	1	0 [6]	Neg	Neg	Relapsed
	603	6	21	1	6 [44]	Neg	Neg	Relapsed
	607	5	14	2	1 [28]	Neg	Neg	Relapsed
	609	6	21	1	8 [8]	Neg	neg	Cured
Median (range)		5.5 (5-6)	21 (7-27)	1(1-8)	5 (0-45)	Cure rate = 7/8 (88%)	Cure rate = 8/8 (100%)	3/8 (37.5%)
II	571	6	21	1	4 [11]	Neg	Neg	Cured
	596	5	7	1	8 [19]	Neg	Neg	Cured
	600	5	14	1	6 [6]	Neg	Neg	Cured
	624	5	7	1	4 [4]	Pos	Neg	Relapsed
	625	5	21	1	1 [20]	WD		
	630	5	21	4	20 [20]	Pos	Neg	Relapsed
	652	5	21	2	10 [11]	Neg	Neg	Relapsed
	653	5	28	2	6 [141]	Neg	Neg	Relapsed
Median (range)		5.0 (5-6)	21 (7-27)	1 (1-4)	6 (1-20)	Cure rate 5/7 (71%)	7/7 (100%)	3/7 (42.5%)

Key: ID = identity in the laboratory; PP = pre-patent period; DPI = days post infection; CSF = cerebrospinal fluid; Tryps = trypanosomes; WC = white cells; EoT = end of treatment; ToC = test of cure; Neg = Negative; Pos = positive; WD = withdrawn from the experiment after 10^{th} drug dose due to toxicity; Numbers in square brackets = maximum number of white cell counts observed during any of the four weekly samplings between 0 -27 DPI; I: DB844 5 mg/kg x 10 days per os; 28-37 DPI; II: DB844 6 mg/kg x 14 days per os; 28-41 DPI

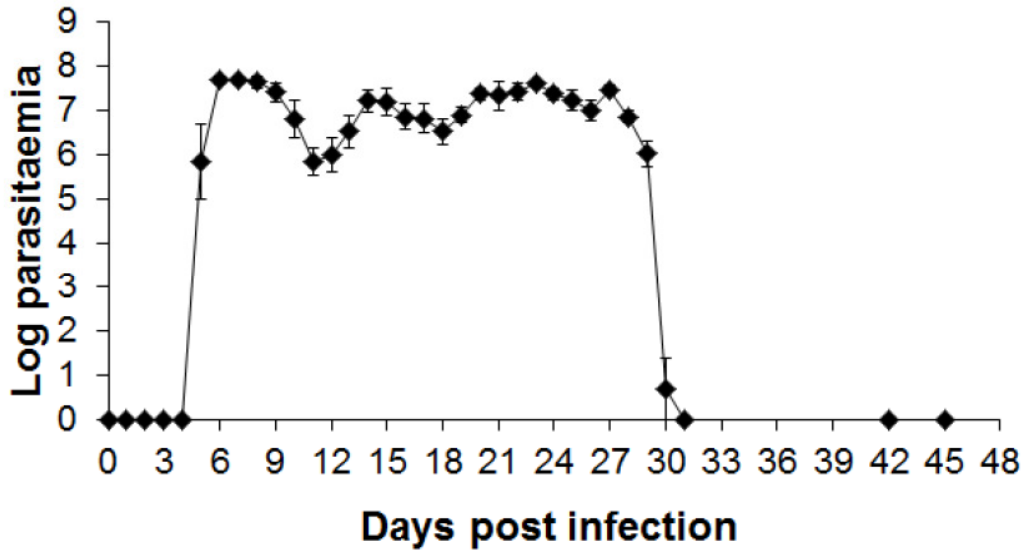

Figure 3: Parasitaemia pattern in monkeys infected with *T.b. rhodesiense* KETRI 2537 and subsequently treated with DB844.

Symbols and error bars represent means and SEs, respectively, of 7 animals; monkeys were treated with DB844 at 6 mg/kg x 14 days, from 28-41 days post infection; Log parasitaemia values were determined by microscopic examination of wet smears of blood using the matching method of Herbert and Lumsden, 1976 [23].

Table 3: Haematologic effects of *T. b. rhodesiense* KETRI2537 infection and treatment with DB844 in vervet monkeys

Parameters	Days post infection (days post last drug dose)					
	0	27	41 (0)	48 (7)	69 (28)	104 (63)
RBC (x 10^6/µl)	5.8 ± 0.2	4.2 ± 0.2, $p < 0.0001$	4.8 ± 0.3, $p < 0.0001$	4.9 ± 0.2, $p < 0.004$	5.5 ± 0.2, $p = 0.55$	6.1 ± 0.3 $p = 0.02$
Hemoglobin (g/dl)	13.4 ± 0.6	9.1 ± 0.6, $p < 0.0001$	11.2 ± 0.7, $p < 0.0001$	11.5 ± 0.6, $p < 0.0001$	12.8 ± 0.5, $p = 0.15$	14.1 ± 0.8, $p = 0.11$
Haematocrit %	43.9 ± 2.0	29.8 ± 1.7, $p < 0.0001$	31.6 ± 1.6, $p < 0.0001$	31.8 ± 1.6, $p < 0.0001$	43.8 ± 1.6, $p = 0.91$	46.7 ± 2.7, $p = 0.91$
Mean corpuscular volume (fl)	78.2 ± 1.5	70.8 ± 0.8, $p < 0.0001$	65.3 ± 0.9, $p = 0.0001$	64.8 ± 0.9, $p = 0.0001$	79.8 ± 1.4, $p = 0.11$	77.0 ± 1.5, $p = 0.21$
Mean corpuscular haemoglobin (g/dl)	23.9 ± 0.4	21.6 ± 0.3, $p < 0.0001$	23.0 ± 0.3 $p < 0.008$	23.3 ± 0.4 $p = 0.07$	23.4 ± 0.6, $p = 0.13$	23.3 ± 0.5 $p = 0.21$
Platelet counts (x 10^3/µl)	346.5 ± 13.1	183.5 ± 24.6 $p = 0.002$	406.5 ± 22.5 $p = 0.05$	390.7 ± 32.4, $p = 0.11$	332.2 ± 11.9, $p = 0.78$	294.3 ± 17.4 $p = 0.55$
WBC counts (x 10^3/µl)	5.8 ± 0.5	3.0 ± 0.4, $p= 0.003$	7.1 ± 1.0, $p= 0.12$	5.6 ± 1.2, $p= 0.9$	5.9 ± 0.8, $p = 0.8$	5.1 ± 0.1, $p = 0.4$

Key: RBC = red blood cells; WBC = White blood cells; g/dl = grams/decilitre; fl = femtolitres; p-values < 0.05 indicate values that were significantly different from pre-infection baseline (day 0) values (Repeated measures Anova with Fishers PLSD post hoc test); Monkey were treated with DB844 at 6 mg/kg x 14 days, from 28-41 days post infection

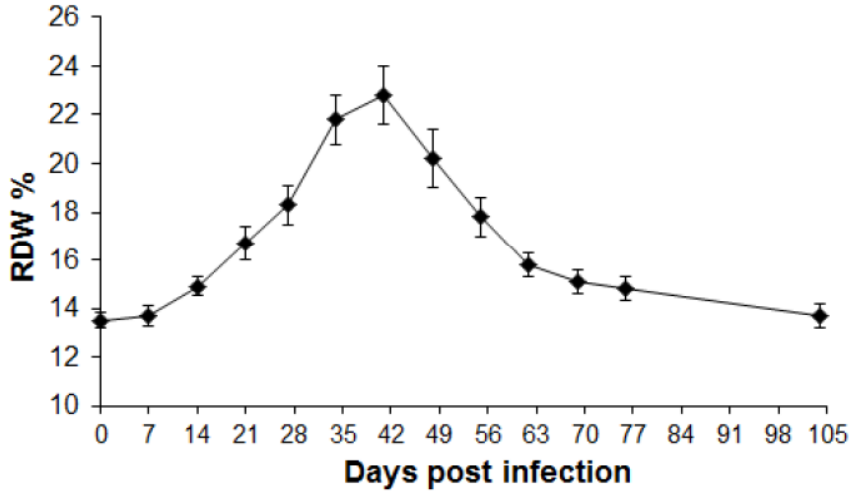

Figure 4: Changes in red cell distribution width in monkeys following infection and subsequent treatment with DB844.

Symbols and error bars represent means and SEs, respectively, of seven monkeys that were treated with DB844 at 6 mg/kg x 14 days, from 28-41 days post infection.

Clinical Chemistries

Plasma from infected monkeys treated with DB844 at 6 mg/kg (group II) was analyzed for several biomarkers of liver and kidney function. Plasma aspartate aminotransferase (AST) did not change significantly following infection but peaked transiently during drug administration (Figure 5, Figure S1). At 24 hours post dosing, mean (± SE) plasma AST increased to 3.3 times above baseline, from 37.0 ± 4.8 IU [95% CI = 26.7-46.8] (day 0) to 121 ± 21.6 IU [95% CI = 65.5 -176.5, p = < 0.0001]. Mean plasma alanine aminotransferase (ALT) exhibited an increasing trend immediately after infection (day 0) and peaked after 7 daily drug doses (34 DPI) (Figure 5). At its peak, mean plasma ALT increased by 2.7 times above baseline, from 4.5 ± 0.9 IU [CI = 2.1-6.9] (day 0) to 13.6 ± 3.8 IU [CI = 3.8-23.4; p = 0.008]. Aberrations in ALT resolved rapidly after treatment. Monkeys further demonstrated a significant infection-related 25.8% decrease in mean plasma albumin concentration, from 35.3 ± 1.9 [CI = 30.4-40.3] g/l at baseline to 26.2 ± 4.2 [95% CI = 15.4-37.0, p = 0.02] g/l at 27 DPI. Plasma albumin concentration stabilized during the treatment period, then decreased transiently

to a nadir at 48 h post dosing (43 DPI, Figure 5). Total bilirubin and direct bilirubin concentrations fluctuated in a pattern comparable to the transaminases (Figure 5), but none of the changes were statistically significant. Mean alkaline phosphatase was 6.3 ±1.9 at baseline and 4.1± 0.8 at 27 DPI (p = 0.25) and showed no significant changes both during and after drug administration. Two indicators of renal function, blood urea nitrogen (BUN) and creatinine, were evaluated. BUN indicated a mild and reversible decrease in kidney function from 48 h post dosing (43 DPI) (Figure 5). Mean plasma concentration of creatinine fluctuated in a similar pattern to BUN, but the change from baseline was not significant (data not shown).

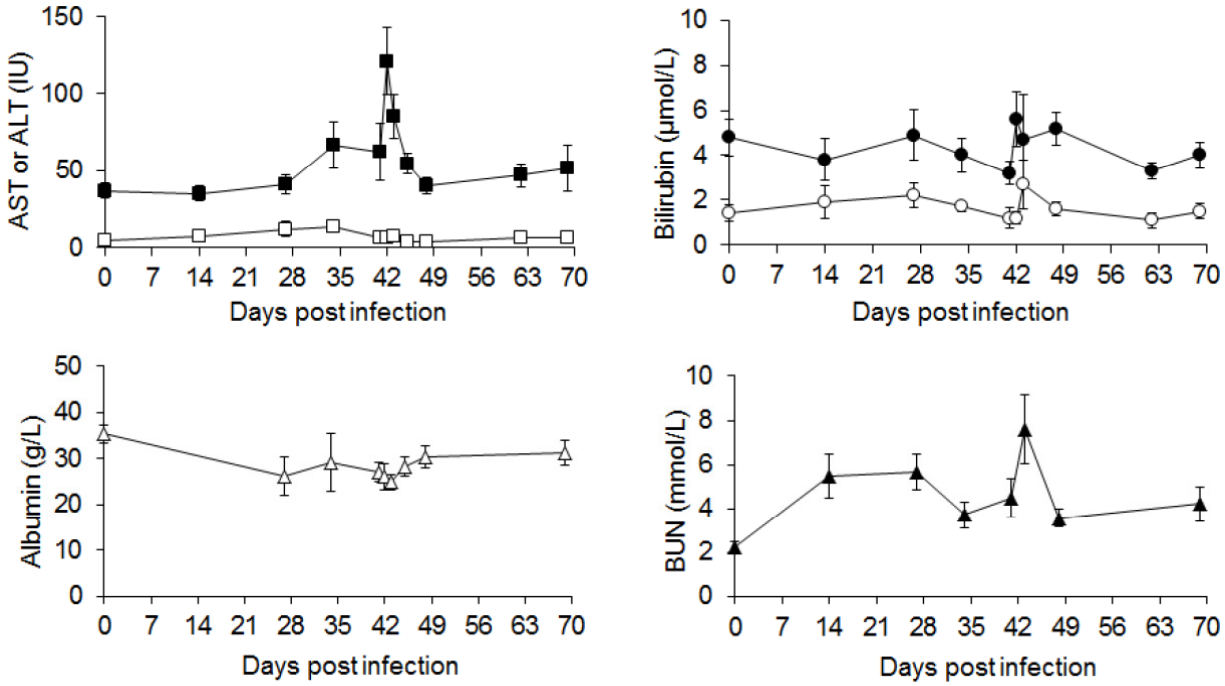

Figure 5: Transient infection and DB844 induced changes in clinical chemistry indicators of liver and kidney function.

Symbols represent mean ± SE (n = 7) of aspartate aminotransferase (AST, ■), alanine aminotransferase (ALT, □), total bilirubin (●), direct bilirubin (0), blood urea nitrogen (BUN, ▲) and albumin (◊); monkeys were treated with DB844 at 6 mg/kg x 14 days, from 28-41 days post infection.

Pharmacokinetics

Plasma and CSF collected from group II monkeys (6 mg/kg) were analyzed for various pharmacokinetic outcomes using traditional non-compartmental methods. After the last (i.e. 14^{th}) dose of DB844, geometric mean (90% CI) concentrations of DB844 peaked at 1 h in plasma with a C_{max} of 430 (100-1800) nM (Table 4, Figure 6) with modest individual animal variations in PK profiles (Figure S2). The active metabolite, DB820, peaked at 4 h in plasma with a C_{max} of 190 (110-320) nM. Exposure to DB820 was three-fold higher than that of DB844, as assessed by the metabolite:parent AUC ratio (Table 4). DB844 concentrations decreased at a faster rate than DB820 concentrations, with a geometric mean (90% CI) apparent terminal elimination half-life of 5.8 (3.4-9.6) h. DB820 was detected in plasma up to 28 days after the last dose of DB844 with a geometric mean (90% CI) concentration of 35 (14-86) nM. Given the protracted decline in DB820 plasma concentrations, the duration of plasma collection was insufficient for accurate estimation of the terminal half-life of DB820 for all monkeys (Figure 6). DB844 was detected in CSF 1 h post dose, with a geometric mean (90% CI) concentration of 17 (7.3-39) nM, and was not detected thereafter. DB820 was not detected in the CSF, with only 2/7 monkeys (monkeys 571 and 624) showing sporadic low concentration (< 4 nM) between 24 and 96 h post dose.

Table 4: Pharmacokinetics of DB844 and DB820 in vervet monkeys after the 14th oral dose at 6 mg/kg (n = 7)

Outcome	Units	DB844		DB820	
AUC(0-∞)	nmol/L•day	70	(16, 290)	NC*	NC
AUC_{last}	nmol/L•day	68	(16, 300)	1400	(760, 2500)
Metabolite: Parent AUC Ratio	NA	--	--	3	(1.1, 6.5)
Cl/F	L/day/kg	240	(56, 1000)	NC	NC
C_{max}	nmol/L	430	(100, 1800)	190	(110, 320)
$t_{1/2}$	day	0.24	(0.14, 0.40)	NC†	NC
T_{max}	day	0.04	(0.04, 0.17)	0.17	(0.08, 0.17)

Key: Values are geometric mean (90% CI) except for metabolite:parent AUC ratio and T_{max}, which are reported as median (range). Metabolite:parent AUC ratio was derived using the AUC from time zero to the last common time point for DB844 and DB820.; *% AUC extrapolated to infinite time >25%. †Duration of sample collection was insufficient to derive an accurate estimate of terminal half-life; AUC(0-∞), AUC from zero to infinite time; AUC_{last}, AUC from time zero to the last measurable concentration; Cl/F, apparent (oral) clearance; C_{max}, maximum concentration; $t_{1/2}$, terminal half-life; T_{max}, time to reach C_{max}; NA, not applicable; NC, not calculable.

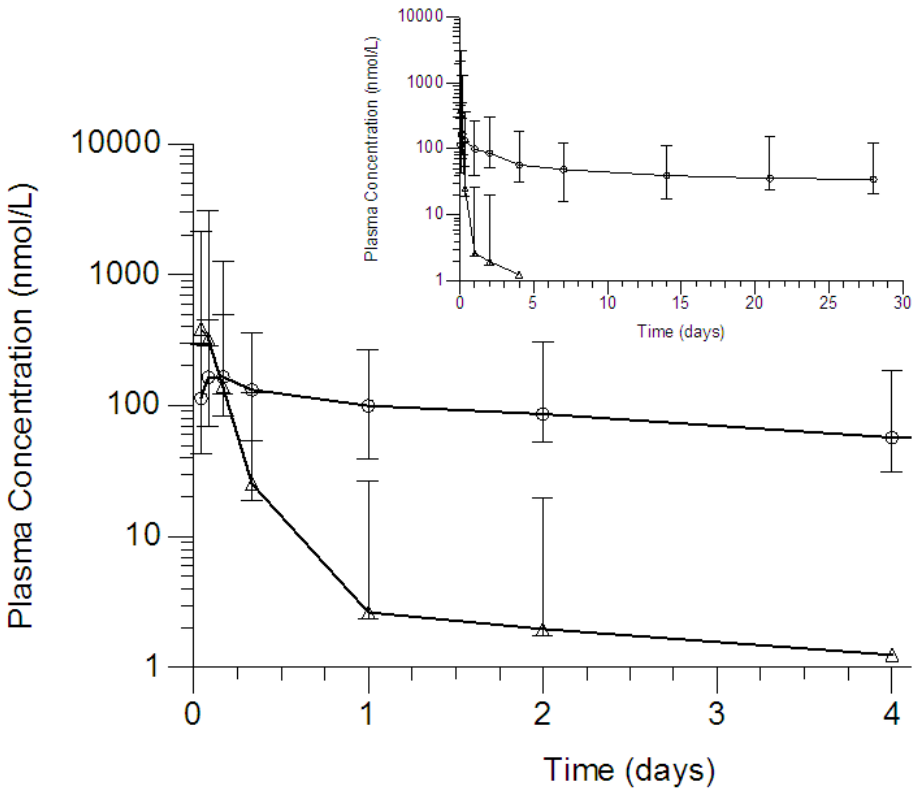

Figure 6: Plasma concentration-time profiles following oral administration of the last (14th) daily dose of DB844.

Symbols and error bars represent geometric means and SEs, respectively, for DB844 (△) and DB820 (○). The monkeys (n = 7) were treated with DB844 at 6 mg/kg x 14 days, from 28-41 days post infection. The insert graph shows the extended profile up to 28 days post the last daily dose of DB844.

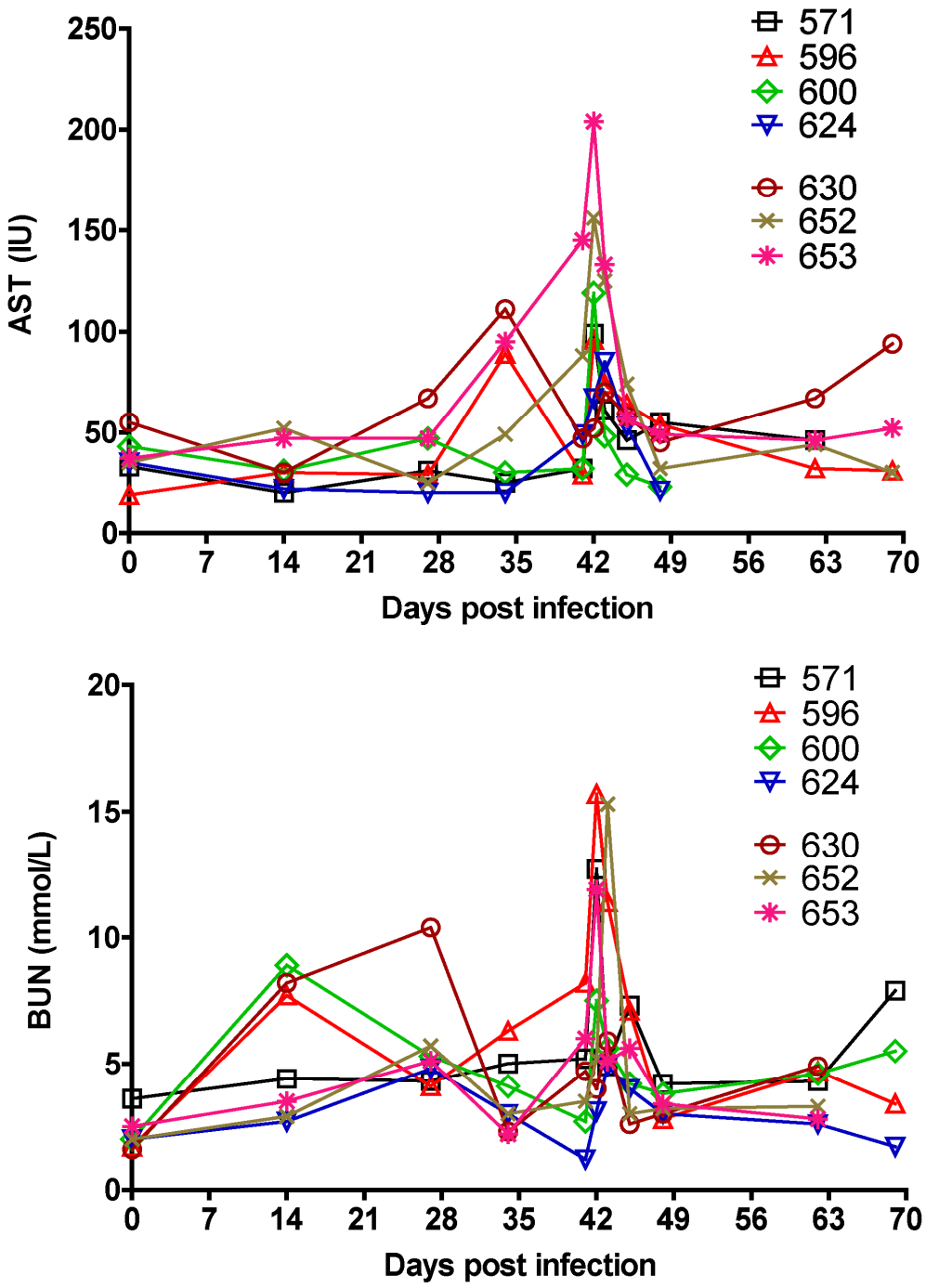

Figure S1: Individual monkey activity/concentration-time profiles of aspartate amino transferase and blood urea nitrogen in plasma.

The monkeys were treated with DB844 at 6 mg/kg x 14 days, from 28-41 days post infection with *T.b. rhodesiense* KETRI2537.

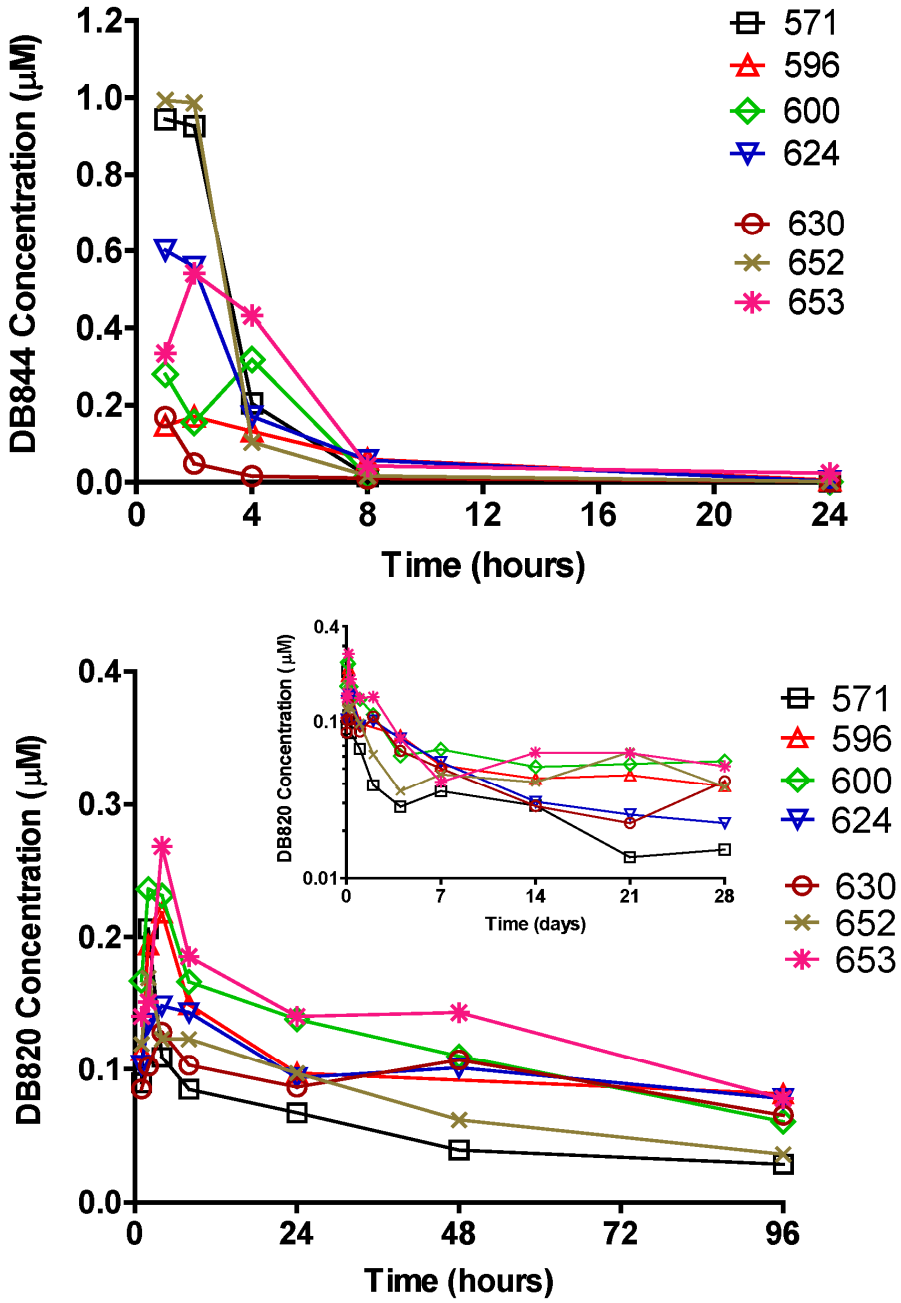

Figure S2: Individual monkey concentration-time profiles of DB844 and DB820 in plasma. The monkeys were treated orally with DB844 at 6 mg/kg x 14 days, from 28-41 days post infection with *T.b. rhodesiense* KETRI2537. The insert graph shows the extended profiles up to 28 days post the last daily dose of DB844

DISCUSSION

The current study has shown that the novel diamidine prodrug (DB844) was effectively metabolized by male vervet monkey liver microsomes to yield at least seven metabolites which were also detected when DB844 was incubated with human liver microsomes [13]. The order in which metabolites were generated in the monkey liver microsomal/drug mixtures and their relative concentrations, dominated by M1A (DB1284) and M1B (DB1058) within the first 20 minutes and by M3 (DB821) thereafter, were also similar to the pattern observed in human liver microsomes [13] suggesting that vervet monkeys would be a useful animal model for evaluation of a drug (DB844) that was in development as a potential therapeutic agent for a human disease (HAT). Our study did not investigate the enzymes responsible for the conversion of DB844 to DB820. It has however been previously shown that conversion of pafuramidine (DB289) to furamidine (DB75) was catalysed by cytochrome P450 enzymes and cytochrome b_5/b_5 reductase in the human liver [22, 29]. Liver microsomes derived from female vervet monkeys and Cynomolgus monkeys metabolized DB844 as efficiently as those derived from male vervets (data not shown), thus justifying evaluation of DB844 in monkeys.

The prodrug (DB844) was well tolerated when tested in uninfected vervet monkeys at the lowest dose (5 mg/kg x 10 days) but was toxic to both monkeys when administered at the highest dose (20 mg/kg x 8 days). The middle dose (10 mg/kg x 10 days) was well tolerated by 1/2 monkeys, suggesting that this dose was slightly more than the maximum tolerated dose in uninfected monkeys. Clinical signs of overt toxicity were detected either after completion of dose regimen (10 mg/kg group) or late into the treatment regimen (20 mg/kg group), suggesting that overt toxicity was dependent on both the daily drug dose and duration of drug administration. These results were consistent with a previous report in which several pentamidine derivatives were associated with acute to chronic toxicity in rodents, which was cumulative in nature with respect to drug exposure [30]. The no observed adverse effects level (NOAEL) for oral DB844 in un-infected monkeys was therefore in the range 5 - < 10 mg/kg, and as a result, daily dose levels of 5 and 6 mg/kg were chosen for the subsequent efficacy study.

DB844/DB820 concentrations in plasma samples from un-infected monkeys (5 mg/kg group), though limited in scope, demonstrated that the prodrug was absorbed after oral administration and converted to the active metabolite (DB820). Similarly, when Cynomolgus monkeys were dosed orally with DB844 at 3 or 10 mg/kg, the drug was absorbed and the resulting plasma DB844/DB820 concentration-time profiles were comparable to those achieved in mice that were dosed at 25 and 100 mg/kg, respectively (Michael Z. Wang, personal communication). These findings were significant since DB844 at 25 or 100 mg/kg was subsequently determined to be curative for 1st and 2nd stage experimental murine HAT infections respectively [11], suggesting that comparatively low dose levels could be efficacious in primates. A period of dosing of 10-14 days was selected for the vervet DB844 efficacy study, partly informed by a report that during clinical trials of the related 1st stage investigational HAT drug DB289, the duration of dosing had to be increased from 5 to 10 days in order to increase efficacy (Sonja Bernhard, Personal communication). In addition, the fact that in humans 2nd stage HAT is treated for 10 days with melarsoprol or NECT) and 14 days with intravenous eflornithine [4] was considered since these dosing periods are partly influenced by the tissue invasive nature of the human infective parasites.

Oral DB844 achieved up to 43% cure rate in the vervet monkey model of 2nd stage HAT. Prior to initiation of treatment at 28 DPI, all 16 monkeys in this study were confirmed to have trypanosomes and pathological white cell numbers in their CSF, confirming that the model fulfilled the criteria for classification of CNS (late, 2nd) stage disease [4]. Pathophysiology studies have shown that trypanosome entry into the CSF initiates meningitis and leads to elevated CSF nitric oxide and IgM concentrations, all further indicating CNS disease [31, 32]. Although occurrence of histologically demonstrable meningoencephalitis may not be guaranteed in the course of primary *T. b. rhodesiense* infections [31, 33], previous studies have shown that when treatment was initiated at 28 DPI in this monkey model, pafuramidine (DB289) and pentamidine did not cure any monkey ([12]; unpublished TRC-KARI data). The data reported in this study, therefore, indicates that DB844 had an improved activity in the CNS stage monkey model compared to the other diamidines.

Biological activity of orally administered chemotherapeutic agents against tissue invasive parasites such as *T. b. rhodesiense* is dependent upon absorption in the gut and attainment of effective exposure levels of the prodrug and/or active metabolites in body fluids and tissues. Pharmacokinetic evaluation of efficacy study (6 mg/kg group) monkeys confirmed that marked DB844 concentration was attained in plasma within 1 hr of the last dose. Peak plasma levels of DB820 were attained within 4 h post last dosing showing that metabolic conversion of DB844 to DB820 was comparable to previous observations in rats and cynomolgus monkeys involving the related prodrug/active metabolite pair, DB289/DB75 [34]. The peak plasma concentrations of the active drug (DB820) were 37 times (190/5.2) higher than the IC_{50} for *T. b. rhodesiense* STIB900; the DB820 concentrations remained at least 19 times (100/5.2) higher than the IC_{50} for *T. b. rhodesiense* STIB900 for at least 48 h post dosing, suggesting that alternative dose regimens in which the drug was dosed once every two days may be sufficient for bloodstream trypanosomes. The plasma C_{max} achieved by DB844 in our study was significantly higher than the 30-35 nM C_{max} values obtained when DB289 was dosed orally to cynomolgus monkey at 5 mg/kg [34]), consistent with similar observations when both DB844 and DB289 were dosed to mice [35].

Detection of DB844 in the CSF indicated BBB penetration and was likely responsible for observed reduction in trypanosome densities in CSF of all monkeys to below the limit of detection for varying periods and eventual cure of 3/7 (43%) group II monkeys. The active metabolite (DB820) was, however, detected only sporadically in CSF of two of the group II monkeys, one of which was eventually cured and one of which was not. The discordance between the CSF PK data and observed activity may result from three possibilities: i) No CSF samples were collected between 1-24 h post dosing. The reduced sampling time points, though justified due to ethical considerations, may have resulted in underestimation of DB820/DB844 in CSF; ii) Some of the intermediate metabolites of DB844, including the monoamidines DB1212, DB1285, M4A and M4B have been shown to have anti-trypanosomal activity in vitro [11]. Unfortunately, these were not quantified in the vervet CSF in our study; iii) trypanosomes were shown to be capable of accumulating DB820 and DB75 up to 15,000 times above

mouse plasma concentrations [36], suggesting that trypanocidal drug concentrations could accumulate and reduce the density of trypanosomes in CSF despite low concentrations of the active metabolite/s.

Trypanosome recrudescence in the CSF preceded that in the blood (133 vs. 261 days), indicating that CSF/CNS remained a major sanctuary/source of relapse trypanosomes despite the improved efficacy of DB844 compared with other diamidines. This was consistent with the generally low CSF: plasma ratio of 1:27 (3.7%) for DB844 at 1 h post last dosing. In addition, and in spite of the limitations highlighted above, that DB820 was hardly quantified in CSF while it was detected in plasma in relatively high concentrations, accounts for the observation that trypanosomes were eliminated more rapidly from the blood than from the CSF (data not shown). An alternative dosing regimen in which higher daily DB844 doses were administered once every two days as discussed above could possibly result into more DB844/DB820 crossing the BBB and improve CNS activity as observed in the GVR 35 mouse model [11]. The rationale for higher daily drug doses would be based on the fact that drug transport across BBB is influenced by concentration dependent passive diffusion, presence of efflux aiding P-glycoprotein transporters or multidrug resistance-associated protein transporter [37, 38]. Importantly, however, structure activity relationship studies need to be continued to identify molecules with superior activity against second stage HAT.

No DB844-related haematological aberrations were observed in the infected monkey model, indicating that low doses (5 and 6 mg/kg) were safe. Erythrocyte and platelet associated parameters recovered rapidly indicating that erythropoesis and thrombocytogenesis remained robust. White blood cells were similarly not adversely affected, suggesting that DB844 was not myelotoxic. However, trypanosome-induced anaemia, thrombocytopenia and leucopoenia were observed. These are common features of experimental *T.b. rhodesiense* infections in monkeys [12, 21, 39] and natural HAT infections in humans [40-42] whose severity is determined by parasite virulence, time lag from infection to therapeutic intervention and individual host differences. The haematology changes before treatment were comparable to those reported in previous

infections with the KETRI2537 stabilate, indicating good reproducibility of the monkey model. Furthermore, resolution of haematology aberrations such as haemoglobin concentration and RDW was clearly related to treatment showing that these were additional indicators of therapeutic efficacy. In un-infected monkeys, the low dose regimen (5 mg/kg x 10 days) similarly did not manifest haematologic toxicity. At high doses (> 10 mg/kg), however, bleeding and haemosiderosis were observed in multiple organs possibly due to damage of endothelial membranes, increased sequestration of damaged erythrocytes and their subsequent destruction by tissue macrophages [43, 44].

Clinical chemistry results (group II, efficacy study) showed modest (2-3 fold) increases in plasma transaminases (AST and ALT) but not alkaline phosphatase, consistent with hepatocellular pathology. Importantly, these elevations in transaminases were reversible within 4-7 days after the last drug dose, indicating that they were likely caused by transient changes in the permeability of hepatocyte cell membranes (rather than necrosis). In contrast high doses (> 10 mg/kg) caused significant hepatotoxicity that was mainly characterized by fatty degeneration (steatosis), focal necrosis, mononuclear infiltration and haemorrhages. Steatosis is a common toxicity of many other drugs, including tetracyclines, corticosteroids, non-steroidal anti-inflammatory drugs and diamidines [45-48]. Steatosis in the un-infected monkeys was likely caused by impairment of mitochondrial fatty acid ß-oxidation causing microvesicular steatosis and resulting in accumulation of lipid vesicles in the cytoplasm of hepatocytes as previously reported for other drugs [45-48].

Analysis of clinical chemistry indicators of renal pathology in efficacy group II (6 mg/kg) monkeys revealed that blood urea nitrogen (BUN) was minimally elevated while plasma creatinine was not, indicating that kidney function was only transiently affected. In contrast, histopathology results from the un-infected monkeys revealed evidence of renal tubular degeneration in the 10 and 20 mg/kg, showing that a more significant renal pathology resulted from the higher drug doses. Similarly, no lesions were seen at low doses in the GIT while moderate to severe gastroenteritis were observed at high doses. Overall therefore, toxicity was dose-dependent and, taken together with results of the

efficacy study, indicated that there was a lack of therapeutic window for DB844. As a result of toxicity, alternative dose regimens to improve efficacy were not attempted.

In summary, this study showed that the prodrug DB844 achieved a moderate cure rate in the vervet monkey model of 2nd stage HAT, which was in contrast with studies in mice in which much higher DB844 doses (ie 100 mg/kg x 5 days) were tolerated and cured all 5/5 mice [11]. This is perhaps the main reason why new chemical entities (NCEs) that are targeted against HAT are preferably evaluated in both rodent and monkey models to obtain a more comprehensive understanding of safety, efficacy and pharmacokinetics before being forwarded for clinical trials in man. Although further development of DB844 against 2nd stage HAT was discontinued due to the lack of a therapeutic window, DB844 has demonstrated that structural modifications of amidines could eventually result in molecules with promising CNS activity at tolerated dose levels. Indeed, novel amidine analogues with better efficacy and safety profiles have been identified and are currently being evaluated in this vervet monkey model of HAT, which will be reported in due course.

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Chapter 6: Safety, Pharmacokinetic, and Efficacy Studies of Oral DB868 in a First Stage Vervet Monkey Model of Human African Trypanosomiasis

[1,8]John K Thuita, [2]Kristina K Wolf, [1]Grace A Murilla, [2]Qiang Liu, [1]James N Mutuku, [3]Yao Chen, [6]Arlene S Bridges, [1]Raymond E Mdachi, [5]Mohamed Ismail, [4]Shelley Ching, [5]David W Boykin, [6]James E Hall, [6]Richard R Tidwell, [2]Mary F Paine, [7,8]Reto Brun and *[3]Michael Zhuo Wang.

[1]Trypanosomiasis Research Centre, Kenya Agricultural Research Institute (KARI-TRC), Kikuyu, Kenya
[2]UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA
[3]Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas, USA
[4]SVC Associates, Inc., Apex, North Carolina, USA
[5]Department of Chemistry, Georgia State University, Atlanta, Georgia, USA
[6]Department of Pathology and Laboratory Medicine, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA
[7]Swiss Tropical and Public Health Institute, Basel, Switzerland
[8]University of Basel, Basel, Switzerland

*Corresponding author: e-mail address: michael.wang@ku.edu

Short Title: Pharmacology of oral DB868 in a HAT monkey model

This chapter is a manuscript under preparation

ABSTRACT

There are no oral drugs for human African trypanosomiasis (HAT, sleeping sickness). A successful oral drug would have the potential to reduce or eliminate the need for patient hospitalization, thus reducing healthcare costs of HAT. The development of oral medications is a key objective of the Consortium for Parasitic Drug Development (CPDD). In this study, we investigated the safety, pharmacokinetics, and efficacy of a new orally administered CPDD diamidine prodrug, 2,5-bis[5-(*N*-methoxyamidino)-2-pyridyl]furan (DB868, CPD-007-10), in the vervet monkey model of first stage HAT. DB868 was well tolerated at a daily dose up to 30 mg/kg for 10 days, a cumulative dose (CD) of 300 mg/kg. Mean plasma levels of biomarkers indicative of liver injury (alanine aminotransferase, aspartate aminotransferase) were not significantly altered by drug administration ($p > 0.05$). In addition, no kidney-mediated alterations in creatinine and urea concentrations were detected. Pharmacokinetic analysis of plasma confirmed that DB868 was orally available and was converted to the active compound DB829, in both uninfected and infected monkeys. Treatment of infected monkeys with DB868 began 7 days post-infection. In the infected monkeys, DB829 attained median C_{max} values that were 12- (3 mg/kg for 7 days), 15- (10 mg/kg for 7 days), and 31-fold (20 mg/kg for 5 days) greater than the IC_{50} (14 nmol/L) against *T. b. rhodesiense* STIB900. DB868 cured all infected monkeys, even at the lowest dose tested (3 mg/kg x 7 days; CD = 21 mg/kg). In conclusion, oral DB868 cured monkeys with first stage HAT at a cumulative dose 14-fold lower than the maximum tolerated dose and thus, should be considered a lead preclinical candidate in efforts to develop a safe, short course (5-7 days), oral regimen for first stage HAT.

Key words: human African trypanosomiasis; prodrug; diamidine; DB868; preclinical candidate; vervet monkeys; efficacy; toxicity; pharmacokinetics; pentamidine.

AUTHORS SUMMARY

Development of orally administered medicines for human African trypanosomiasis (HAT) would potentially reduce the need for hospitalization of patients, thus lowering the cost of healthcare. In this study we investigated the potential of a novel diamidine prodrug, DB868 (CPD-007-10), to become an oral treatment for first stage HAT. When administered to uninfected monkeys by oral gavage, DB868 was well tolerated up to a maximum dose of 30 mg/kg/day for 10 days (total dose: 300 mg/kg). The compound (DB868) was confirmed to be absorbed from the monkeys' alimentary canal and was successfully converted to the active compound (DB829) in concentrations that were potentially therapeutic for blood trypanosomes. Subsequently, DB868 was evaluated for efficacy in the first stage vervet monkey model of HAT in which treatment was initiated at seven days post infection with *T. b. rhodesiense* KETRI2537. All the infected monkeys were cured, even at the lowest of the three dose regimens that were evaluated, 3 mg/kg/day for seven days (total dose 21 mg/kg), 10 mg/kg/day for seven days (total dose 70 mg/kg) and 20 mg/kg/day for 5 days (total dose 100 mg/kg). Analysis of plasma collected from the infected monkeys for drug levels revealed absorption of DB868 from the gut and subsequent conversion to the active compound (DB829) was comparable in both uninfected and infected monkeys. In view of its good safety and oral efficacy profile, we conclude that DB868 is a suitable candidate for development of a new treatment for first stage HAT.

INTRODUCTION

Human African trypanosomiasis (HAT, sleeping sickness) is caused by two trypanosome species that are transmitted through the bite of blood-sucking tsetse flies (*Glossina spp). Trypanosoma brucei (T. b.) gambiense* is endemic to West and Central Africa, while *T. b. rhodesiense* is endemic to East and Southern Africa [1]. The disease is focal in distribution and is marked by wide temporal and spatial variations in incidence and prevalence [2-4]. HAT is characterised by two clinical stages. During the first (early, haemolymphatic) stage, trypanosomes proliferate at the site of the fly bite, travel to local lymph nodes and bloodstream, and progressively invade other tissues [5]. Approximately 3-4 weeks post-infection with *T. b. rhodesiense,* or months to years with *T. b. gambiense*, trypanosomes invade the central nervous system (CNS), initiating the second (late, meningo-encephalitic) stage of HAT [5]. Second stage HAT is marked by neurological and endocrine disorders. If patients are not treated, they lapse into coma and die [6].

HAT chemotherapy is stage specific. Only two drugs have been approved for the treatment of first stage HAT, pentamidine and suramin. Pentamidine, a diamidine first used clinically in 1941, is used to treat first stage *T. b. gambiense* infections. Suramin, a naphthylurea first introduced for clinical use in 1921, is effective against both trypanosome species but is mainly used against first stage *T. b. rhodesiense* HAT [7,8]. Pentamidine is associated with hypoglycaemia, pain at the injection site, diarrhoea, nausea and vomiting, while suramin is associated with hypersensitivity reactions, albuminuria, haematuria and peripheral neuropathy [7]. In addition, these drugs must be administered *via* intramuscular injection or intravenous infusion in well-equipped hospitals, which are not readily available or accessible in rural areas where HAT typically occurs. To overcome these limitations, two orally active compounds, fexinidazole and the oxaborole SCYX-7158, have recently entered clinical development to treat both stages of the disease [9]. In addition, efforts by the Consortium for Parasitic Drug Development (CPDD) to address these limitations have resulted in the synthesis of a collection of diamidines with promising pharmacologic properties [10,11].

One of the new aza diamidines, 2,5-bis(5-amidino-2-pyridyl)furan (DB829; Figure 1), exhibited an IC_{50} of 14 nmol/L against *T. b. rhodesiense* STIB900 *in vitro* [12]. In addition, it was shown to be 100% curative in both the acute (*T. b. rhodesiense* STIB900) and chronic CNS (*T. b. brucei* GVR35) mouse models of HAT after intraperitoneal administration [12]. However, the dicationic nature of DB829 and other diamidines (*e.g.*, pentamidine and furamidine) contributes to poor permeation through biologic membranes and in turn, poor systemic exposure after oral administration [13]. As such, a prodrug of DB829 was designed, 2,5-bis[5-(*N*-methoxyamidino)-2-pyridyl]furan (DB868; Figure 1), by masking the cationic functionalities of the active compound with methoxy groups [14]. Oral administration of DB868 was 100% curative in both the acute and chronic CNS mouse models of HAT [12]. Based on these desirable properties, DB868 progressed into our vervet monkey model to assess its potential as a new lead compound for oral treatment of first stage HAT. The purpose of this study was to evaluate DB868 metabolism in monkey liver microsomes, safety in uninfected monkeys, pharmacokinetics in both uninfected and infected monkeys, and efficacy in infected monkeys.

MATERIALS AND METHODS

Ethics

This study was conducted in accordance with experimental guidelines and procedures (Ref: C/TR/4/325/116) approved by the Institutional Animal Care and Use Committee (IACUC) at the Kenya Agricultural Research Institute's Trypanosomiasis Research Centre (KARI-TRC). These IACUC regulations conformed to national guidelines provided by the Kenya Veterinary Association.

Trypanocidal Test and Comparator Drugs

The test drug, 2,5-bis[5-(*N*-methoxyamidino)-2-pyridyl]furan diacetate (DB868; CPD-007-10; Lot #2-JXS-28; Base MW = 366.37; FW = 564.37), was supplied by the University of North Carolina-led CPDD as a yellow powder stored in opaque, water tight bottles. In the laboratory, the drug-containing vials were wrapped in aluminium foil as further protection from light and stored at room temperature. The drug was dissolved fresh daily in distilled de-ionised water (pH 4.5 ± 0.2) at concentrations permitting administration of 2 mL/kg body weight per oral administration. For example, a 15 mg/mL dose solution was prepared for animals receiving a dose of 30 mg/kg and a 10 mg/mL dose solution for a dose of 20 mg/kg. Pentamidine isethionate, supplied by the World Health Organization (WHO), was used as the comparator drug. Pentamidine was dissolved in sterile distilled water and administered intramuscularly at 0.5 mL/kg body weight.

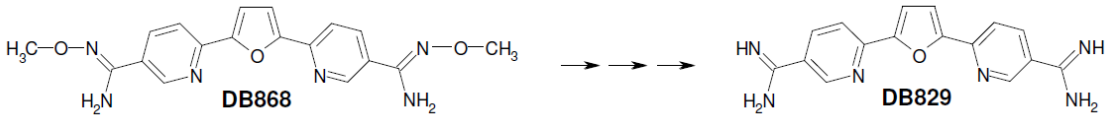

Figure 1. Structures of the prodrug (DB868) and active compound (DB829).

Experimental Animals

Eighteen vervet monkeys, also known as African green monkeys or *Chlorocebus (Cercopithecus) aethiops*, weighing from 2.0 to 4.5 kg, were acquired from the Institute of Primate Research in Kenya. To ensure animal welfare and ameliorate suffering, upon arrival at KARI-TRC, the monkeys were subjected to standard quarantine procedures, including screening for zoonotic and non-zoonotic diseases/infections and treatment for both endo- and ectoparasites, for a minimum of 90 days prior to study commencement as previously described [15,16]. They became accustomed to staying in individual squeeze-back stainless steel cages during this time. The monkeys were maintained on a diet of fresh fruits and vegetables (bananas, tomatoes, carrots and green maize) and commercial monkey cubes (Unga Feeds®, Nakuru, Kenya) fed twice daily, and were given water *ad libitum*. The commercial monkey cubes were manufactured to have the following nutrient composition: crude protein, 19.4% (w/w); crude fiber, 5.6% (w/w); ether extracts that include fats and lipids, 4.2% (w/w); and nitrogen-free extracts, 66.5% (w/w).

Metabolism of DB868 in Vervet Monkey Liver Microsomes

DB868 metabolism was studied in male vervet monkey liver microsomes (custom-prepared by XenoTech, LLC, Lenexa, KS, USA) by adapting a previously published method [17]. Briefly, incubation mixtures contained 10 μM DB868, 0.5 mg/mL monkey liver microsomes, and 3.3 mM $MgCl_2$ in 100 mM phosphate buffer (pH 7.4). Reactions were initiated by the addition of NADPH (1 mM final concentration). Control incubations were carried out without NADPH, DB868, or liver microsomes. Aliquots (100 μL) of the reaction mixtures were removed at 0, 5, 10, 15, 30, 60 and 120 min and mixed with 100 μL of ice-cold acetonitrile. After centrifugation to pellet precipitated proteins, the supernatants were analyzed by HPLC/UV and fluorescence using the method previously described for pafuramidine (DB289) and furamidine (DB75) [18]. Metabolite identification was performed by comparing retention times to those of synthetic standards for M1 (DB1679), M2 (DB840), M3 (DB1712), and DB829. DB868 and its metabolites were quantified using a calibration curve (0.1 – 10 μM) generated using synthetic standards.

Safety and Pharmacokinetics in Uninfected Monkeys

Eight uninfected vervet monkeys, divided into two dose-groups of four monkeys (two females and two males) each, were used. Baseline weight and clinical and haematological data were collected over a 14-day period, after which the monkeys were orally administered DB868 at 10 mg/kg/day (group 1) or 30 mg/kg/day (group 2) for 10 days (Table 1). Care was taken to avoid spillage and to minimise the time between drug preparation and dosing. Drug administration utilized a dose volume of 2 mL/kg body weight. The animals were monitored for indicators of overt toxicity, including changes in feed intake, weight, demeanour, posture and stool composition and consistency. Feed intake was assessed by scoring the proportion of the daily ration consumed by each monkey on a scale of 1 (full ration eaten), ¾, ½, ¼, and 0 (no feed eaten) as previously described [15]. To increase chances of detecting potential drug-related gastrointestinal toxicity, stool samples were collected and examined visually and by faecal occult blood tests conducted according to the modified guaiac method [19]. Post-last drug dose (LDD) monitoring extended to a minimum of 60 days.

During pre- and post-dose monitoring, monkeys were anaesthetized by intramuscular injection of ketamine HCl (10-15 mg/kg) to facilitate physical examination, body weight measurements, and sample collection. Blood was collected from the femoral vein *via* inguinal venipuncture as described previously [16] and divided into aliquots: 1 mL blood into EDTA-containing tubes (1.5 mg EDTA/mL blood) for full haemogram determination and 2 mL blood into EDTA-containing tubes for plasma separation. Plasma was separated using a cool spin centrifuge (1500 rpm for 10 min at 4°C). The harvested plasma was divided into aliquots for clinical chemistry determinations (500 µL), prodrug (DB868) and active compound (DB829) concentration measurement (150 µL), and preservation as a stock sample (approximately 250 µL). All plasma aliquots were frozen at -20°C before analysis.

Efficacy and Pharmacokinetics in Infected Monkeys

The efficacy of DB868 administered orally at 20 mg/kg/day for 5 days was compared to that of pentamidine administered intramuscularly at 4 mg/kg/day for 7 days.

To obtain an indication of dose response, DB868 also was evaluated at 10 and 3 mg/kg/day, administered orally for 7 days. The 10 and 3 mg/kg dose regimens were evaluated at a time when a regulator-imposed freeze on the acquisition of non-human primates was in force, hence only 2 animals were available per dose group. In each experiment, a 14-day baseline data collection period was observed, after which the monkeys were infected by intravenous injection of 10^4 *T. b. rhodesiense* KETRI 2537 trypanosomes diluted from the infected blood of immuno-suppressed donor Swiss white mice [16]. Post-infection monitoring for the development of parasitaemia was initiated at three days post-infection (DPI) while treatment began 7 DPI, subsequent to confirmation of first stage HAT (defined as trypanosomes detectable in blood and not cerebrospinal fluid [CSF], and CSF white cell counts less than 5 cells/mm^3) [16]. Ear-prick blood samples to determine parasitaemia were collected prior to daily drug administration. Clinical and parasitological cure was evaluated for at least six months as previously described [16]. In our studies with the related prodrug DB844, plasma samples collected out to 28 days post-LDD (6 mg/kg) were insufficient to recover a robust estimate of the elimination half-life of the active compound DB820 [20]; therefore, in the current study, plasma samples were collected for at least 60 days post-LDD for pharmacokinetic analysis. Haematology samples were analysed using an AC^{3diff}T Coulter Counter (Miami, FL, USA). Clinical chemistry determinations were performed using a Humalyzer analyser system. Plasma was analyzed for prodrug and active compound concentrations using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) as described below.

Handling of Moribund Infected Monkeys

Monkeys that were deemed as treatment failures/relapses or developed severe adverse clinical signs as defined in the protocol (*e.g.*, inability or reluctance to perch, less than ¼ of normal daily feed intake for 2-3 consecutive days) were immediately withdrawn from the study and humanely euthanized for post-mortem examination. These monkeys were euthanized by intravenous administration of 20% (w/v) pentobarbitone sodium solution (150 mg/kg body weight; Euthatal®; Rhône-Mérieux, United Kingdom).

HPLC-MS/MS Quantification of DB868 and DB829 in Monkey Plasma

Monkey plasma samples were processed for quantification of DB868 and DB829 using previously described methods [21,22] with modifications made to the transition and mass spectrometer parameters. DB868-d_6P (DB868 with deuterated pyridyl rings; 30 nM) and DB829-d_6 (DB829 with deuterated pyridyl rings; 30 nM) were used as internal standards and were supplied by the CPDD. Prodrug and active compound were separated on an Aquasil C_{18} HPLC column (50 × 2.1 mm, 5 µm; Thermo Fisher Scientific, Waltham, MA, USA) and quantified using an Applied Biosystems API 4000™ triple quadrupole mass spectrometer equipped with a Turbo V™ source and electrospray probe (Foster City, CA, USA). The following transitions (in positive ion mode) were used in multiple reaction monitoring scans: 367.1→320.2 (DB868), 373.7→323.2 (DB868-d_6P), 307.1→290.1 (DB829), and 313.2→296.2 (DB829-d_6). Calibration standards and quality controls were prepared in blank monkey plasma to mimic the matrix of the unknown test samples. Analyte concentrations were reported only for those samples that were between the standards and controls that had an accuracy and precision within 100% ± 20%. If samples were below this range, data are reported as below the limit of quantification. Data below the limit of quantification were not used for the pharmacokinetic analysis.

Data Analysis

Data were analysed statistically using StatView for Windows Version 5.0.1 (SAS Institute Inc., Cary, NC, USA) as previously published [20]. Repeated measures ANOVA, with Fisher's PLSD post hoc test, was used to test the effects of trypanosomal infection, as well as DB868, on haematological and clinical chemistry parameters in comparison with respective baseline values ($\alpha = 0.05$). Confidence intervals (95%) were derived to further test the significance of observed findings. The clinical data arising from the efficacy study are presented descriptively since the group sizes were too small for statistical analysis. Pharmacokinetic outcomes were determined with standard non-compartmental methods using Phoenix WinNonlin (version 6.2; Pharsight, Mountain View, CA, USA).

RESULTS

Conversion of Prodrug to Active Compound in Monkey Liver Microsomes

The prodrug DB868 was metabolized in male vervet monkey liver microsomes to M1 (DB1679), M2 (DB840), M3 (DB1712), M4, and the active compound DB829 (Figure 2). These metabolites were similar to those observed when DB868 was incubated with human liver microsomes [23]. Detection of these metabolites indicated that, like pafuramidine (DB289) and DB844, DB868 undergoes sequential *O*-demethylation and *N*-dehydroxylation reactions to form the active compound DB829 in monkey liver microsomes. M2 (DB840), a bis-amidoxime metabolite, had the highest concentration at the end of the 120-min incubation. DB829 was at the limit of detection in UV mode; however, formation was confirmed by subsequent parallel fluorescence and mass spectrometric detection (data not shown).

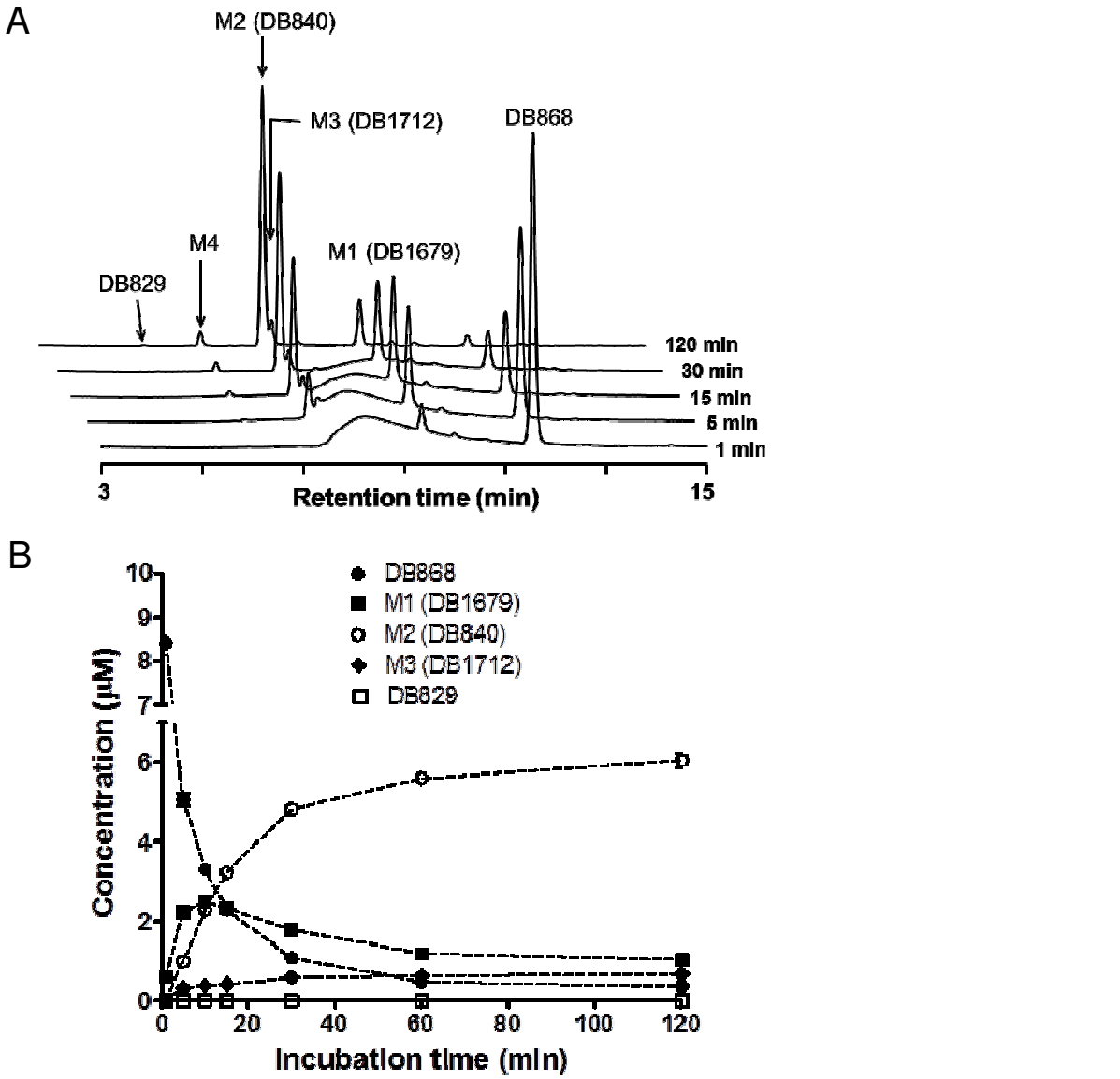

Figure 2. HPLC/UV chromatograms (A) and concentration-time profiles (B) of the prodrug DB868, intermediate metabolites, and active compound (DB829) following incubation of DB868 with male vervet monkey liver microsomes.

Incubation mixtures (1 mL at pH 7.4) contained 10 μM DB868, 0.5 mg/mL monkey liver microsomes, and 1 mM NADPH. Aliquots were removed at 1, 5, 15, 30 and 120 min, and analyzed for DB868, three intermediate metabolites (M1, M2, M3), and DB829 by HPLC/UV. Metabolite M4 was not quantified due to the lack of a synthetic standard. Symbols and error bars represent means and SDs of triplicate incubations.

Overt Toxicity and Haematology in Uninfected Monkeys

Uninfected monkeys administered DB868 orally at 10 mg/kg/day for 10 days (n = 4) did not exhibit any adverse clinical signs throughout the study. In addition, two of the four monkeys in the 30 mg/kg/day group did not display overt toxicity. The remaining two monkeys exhibited mild signs of toxicity, including excess mucous in the stool (monkey 567) and transient inappetance 1-2 days post-LDD (monkeys 567 and 546). In general, stool texture and consistency was unchanged and faecal occult blood tests revealed nothing significant in any study subject, suggesting that no notable gastrointestinal toxicity occurred. The mean body weight of monkeys in the 30 mg/kg group exhibited minimal variation (Figure 3), with a maximum decline of 6.5% from baseline (3.1 kg ± 0.3). A maximum decline of 6.2% from baseline (3.2 kg ± 0.5) was observed in the 10 mg/kg group (data not shown). Overall, the two oral DB868 dose regimens were well tolerated.

Haematological parameters of the two treatment groups did not vary significantly from baseline throughout the study. For the 30 mg/kg group, the baseline mean red blood cell (RBC) and platelet counts (± SE) were 5.5 (± 0.1) x 10^6 and 3.7 (± 0.2) x 10^5 cells/μL of blood, respectively, and showed little change throughout the study (p = 0.10 and 0.06, respectively; Figure 3). Similarly, no significant variations were seen in the RBC or platelet counts for the 10 mg/kg group (data not shown). The mean white blood cell (WBC) count exhibited a minor transient increase post-LDD (1.5-fold over the baseline count (± SE) of 4.8 (± 0.5) x 10^3 cells/μL of blood; p = 0.05; Figure 3), which returned to baseline after 24 h. A comparable trend was seen in the WBC count of the 10 mg/kg group (data not shown), as well as in a separate monkey that was not administered drug or vehicle but had blood sampled at the same time as monkeys in the current experiment, suggesting that the change in the WBC count was not drug-related.

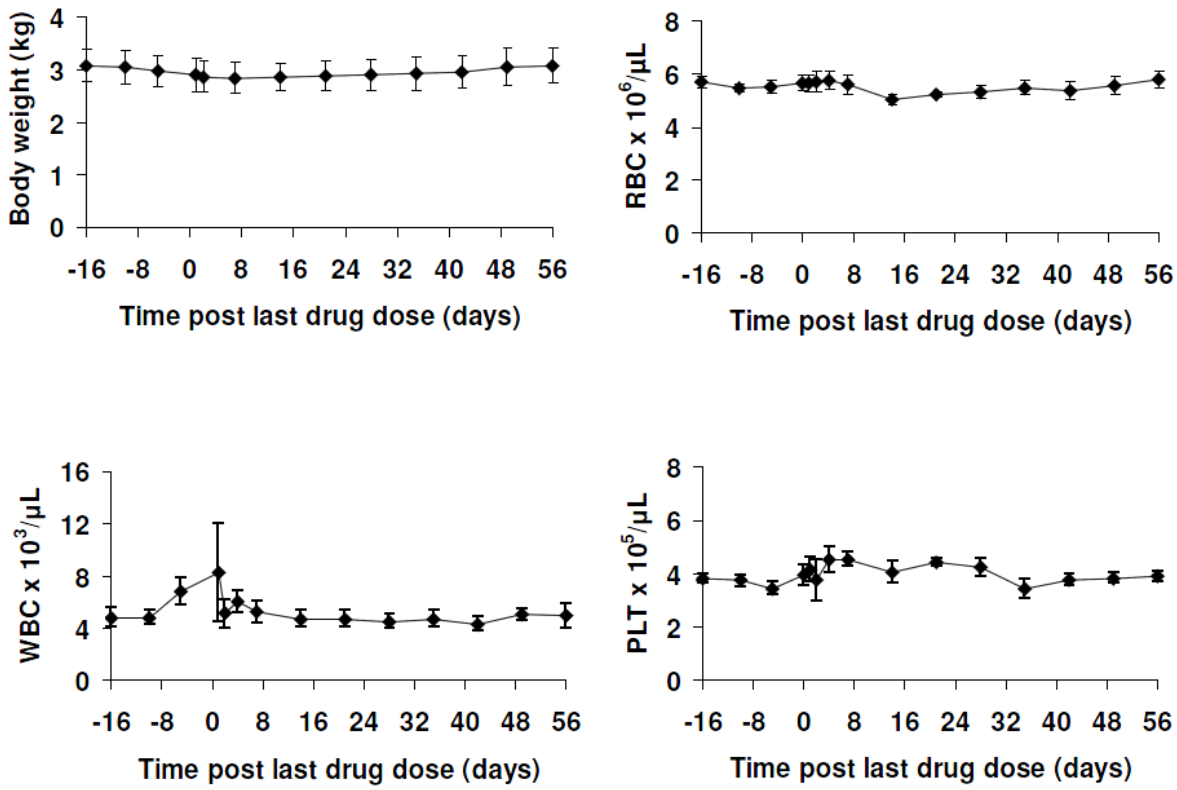

Figure 3. Changes in body weight and haematological parameters in un-infected vervet monkeys administered DB868.

The monkeys (n = 4) were administered DB868 orally at 30 mg/kg/day for 10 days, between day -9 to day 0 post-last drug dose. Symbols and error bars represent means and SEs, respectively, of body weight, red blood cell count (RBC), white blood cell count (WBC), and platelet count (PLT).

Clinical Chemistry

Plasma biomarkers of liver injury, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total and direct bilirubin, were monitored in uninfected monkeys prior to (baseline), during, and following completion of the 10-day DB868 dosing regimens. At the two baseline time points (-16 and -10 days post-LDD), mean (± SE) ALT levels were 14.6 (± 3.1) and 11.1 (± 2.3) IU/L, respectively, for the 10 mg/kg group and 27.3 (± 13.0) and 16.0 (± 7.2) IU/L for the 30 mg/kg group. During and

following the completion of dosing, mean ALT levels varied considerably in the 10 mg/kg group (Figure 4A). The highest post-treatment mean ALT values observed were 2.8-fold (31.3/11.1) greater than the second baseline sample for the 10 mg/kg group and 1.3-fold (20.5/16.0) for the 30 mg/kg group. Neither variation was statistically significant ($p = 0.71$, 10 mg/kg group; $p = 0.37$, 30 mg/kg group). Mean AST and total and direct bilirubin levels also exhibited variability prior to, during, and following dosing (Figure 4, B and C) but overall, were not statistically different from baseline values ($p > 0.05$).

Two biomarkers of kidney injury, creatinine and urea, were also evaluated in plasma samples. Mean (± SE) creatinine concentrations at the two baseline time points were 63.6 (± 4.0) and 54.1 (± 5.5) µmol/L, respectively, for the 10 mg/kg group and 61.5 (± 14.5) and 57.3 (± 6.0) µmol/L for the 30 mg/kg group. Post-dosing variations were minimal (Figure 5A) and not statistically significant ($p > 0.05$). However, mean urea levels exhibited a transient increase following the completion of each dosing regimen (Figure 5B; Figure S1). Plasma urea peaked 1-2 days post-LDD and was 2.1- and 2.7-fold greater than baseline for the 10 mg/kg and 30 mg/kg groups, respectively (both $p < 0.05$). To determine whether increases in plasma urea levels were due to kidney dysfunction, the blood urea nitrogen (BUN):creatinine ratio was calculated for the 1-2 days post-LDD period. The peak mean urea concentration for the 10 mg/kg group was 12.8 mmol/L, which is equivalent to 35.9 mg/dL of BUN. The mean creatinine concentration was 72.3 µmol/L (equivalent to 0.8 mg/dL). Therefore, the BUN:creatinine ratio was 45:1 (35.9:0.8). The BUN:creatinine ratio for the 30 mg/kg group was higher at 48:1.

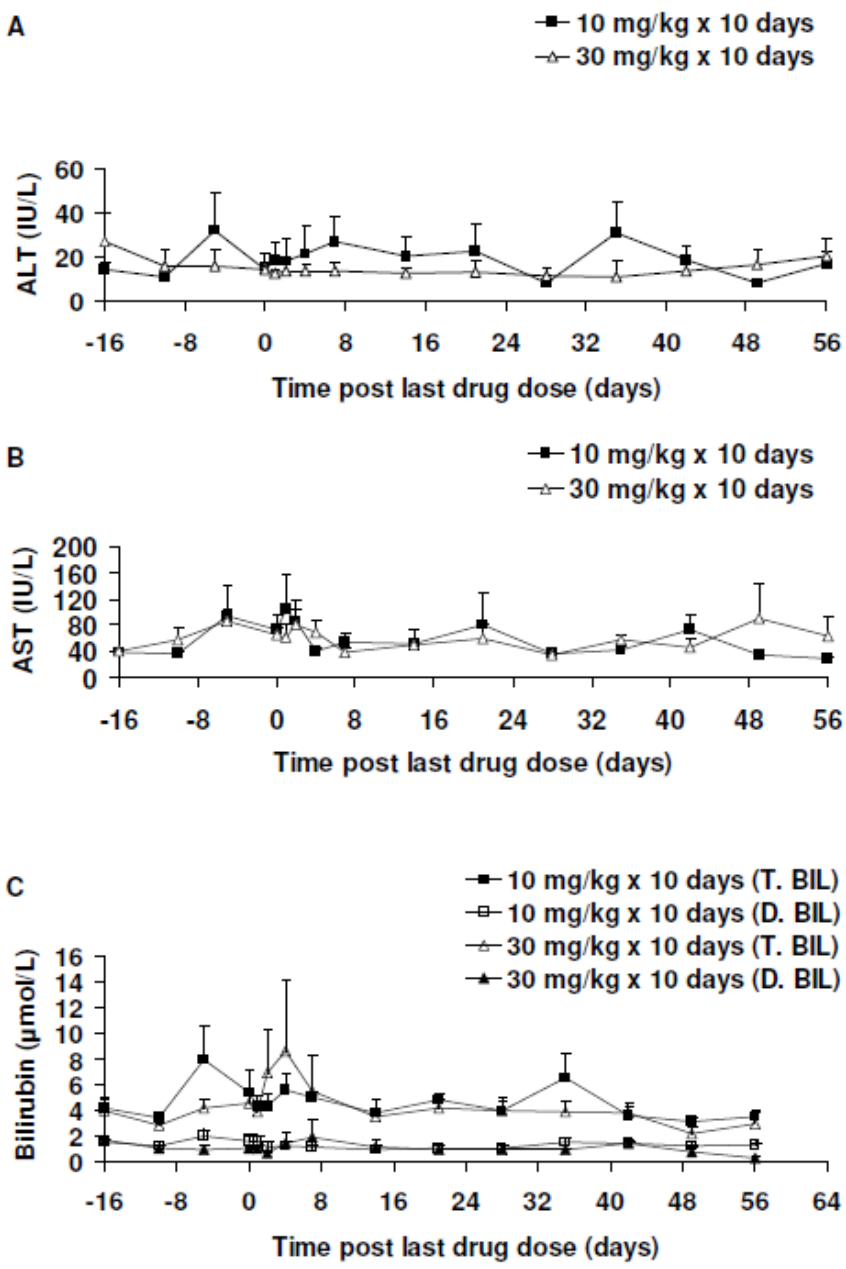

Figure 4. Changes in plasma biomarkers of liver injury in un-infected vervet monkeys administered with DB868.

The two groups of monkeys (each n = 4) were dosed with DB868 daily from -9 to 0 days post last drug dose. Symbols and error bars represent means and SEs, respectively of (A) Alanine aminotransferase (ALT), (B) Aspartate aminotransferase (AST) and (C) Total (T) and direct (D) bilirubin (BIL) levels.

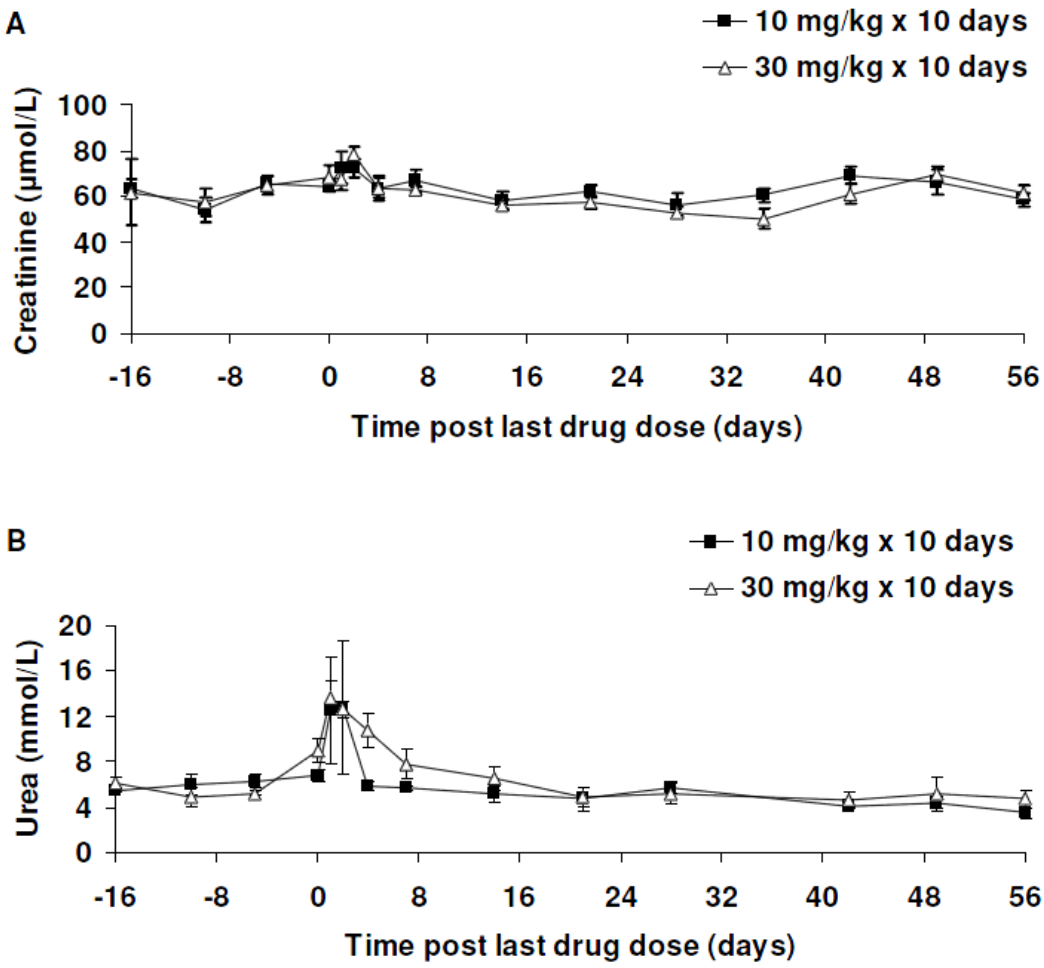

Figure 5. Changes in plasma biomarkers of kidney injury in un-infected vervet monkeys administered with DB868.

DB868 was administered orally at 10 mg/kg/day (n = 4) or 30 mg/kg/day (n = 4) for 10 days, between day -9 to day 0 post-last drug dose. Symbols and error bars represent means and SEs, respectively of (A) creatinine and (B) urea.

Pharmacokinetics in Uninfected Monkeys

Following oral administration of the prodrug DB868 to uninfected monkeys, DB868 was detected in plasma at 4 h post-LDD (Figure 6; Table 1). DB868 concentrations declined to below the limit of detection (BLD) within 1-2 days post-LDD for the 30 mg/kg group and in less than 1 day post-LDD for the 10 mg/kg group (data not shown). Accurate recovery of pharmacokinetic outcomes for DB868 was precluded for the 10 mg/kg dose group (Table 1). Greater inter-individual variability was observed for the 4 h post-LDD concentration (C_{4h}) of DB868 than for DB829 (Table 1). The geometric mean DB868 C_{4h} for the 30 mg/kg group (466 nmol/L) was 5.2-fold greater than that for the 10 mg/kg group (89 nmol/L). The geometric mean DB829 C_{4h} for the 30 mg/kg group (320 nmol/L) was 1.6-fold greater than that for the 10 mg/kg group (185 nmol/L). The geometric mean AUC_{last} and $AUC_{0-\infty}$ for DB829 in the 30 mg/kg group were 2.2-fold greater than that in the 10 mg/kg group. The geometric mean terminal elimination half-life for DB829 was comparable between the two dose groups (29 and 31 days, respectively).

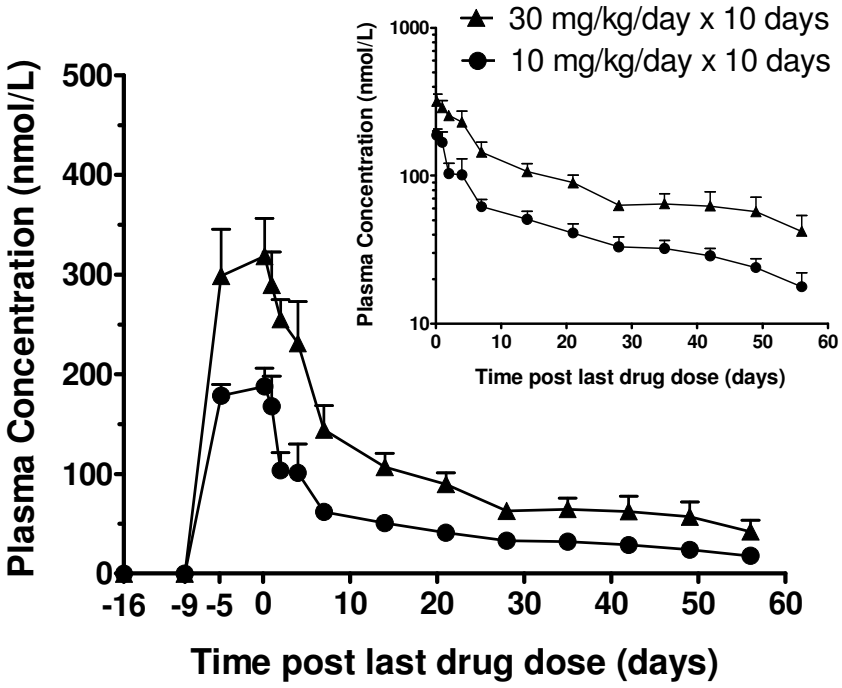

Figure 6. Plasma concentration-time profiles of the active compound DB829 following administration of the prodrug DB868 to un-infected vervet monkeys.

DB868 was administered orally at 10 mg/kg/day (n = 4) or 30 mg/kg/day (n = 4) for 10 days, between day -9 to day 0 post last drug dose. Symbols and error bars represent geometric means and SEs, respectively. The inset graph shows the plasma concentration-time profiles starting at day 0 post-last drug dose on a logarithmic scale.

Table 1. Pharmacokinetics of DB868 and DB829 in un-infected vervet monkeys after the final (10^{th}) oral dose of DB868

Compound	Outcome	Units	Oral DB868							
			10 mg/kg x 10 days				30 mg/kg x 10 days			
		Monkey ID	643	659	675	677	546	567	668	679
DB868	C_{4h}	nmol/L	50	130	39	250	1600	280	320	330
	C_{24h}	nmol/L	BLQ	BLQ	6	BLQ	38	47	35	66
	AUC_{last}	nmol/L•day	NC	NC	NC	NC	530	210	170	190
	$AUC_{0-\infty}$	nmol/L•day	NC	NC	NC	NC	540	260	180	200
	$t_{1/2}$	day	NC	NC	NC	NC	1	1	1.3	0.4
DB829	C_{4h}	nmol/L	240	160	180	170	290	300	270	440
	C_{24h}	nmol/L	140	260	120	170	210	280	340	360
	AUC_{last}	nmol/L•day	3000	2800	2100	2100	5700	4600	7100	4700
	$AUC_{0-\infty}$	nmol/L•day	3700	3700	4100	2400	7000	6200	12100	5600
	$t_{1/2}$	day	30	30	55	18	24	29	47	22

Key: C_{4h}, concentration at 4h; C_{24h}, concentration at 24 h; AUC_{last}, area under the curve from time zero to the last measurable concentration; $AUC_{0-\infty}$, area under the curve from time zero to infinite time; $t_{½}$, terminal elimination half-life; BLQ, below limit of quantitation; NC, not calculable.

Disease Progression and Efficacy

Following inoculation, the median (range) prepatent period of *T. b. rhodesiense* infection in the monkeys was 4.5 (3-6) days (Table 2; Figure 7). The bloodstream form of *T. b. rhodesiense* KETRI 2537 trypanosomes multiplied rapidly, reaching a peak mean count of 1.1 x 10^7 trypanosomes/mL blood; in some monkeys, the count peaked as high as 1.3 x 10^8 trypanosomes/mL (antilog 8.1; Table 2). Classical signs of *T. b. rhodesiense* infection were observed, including rough hair coat, dullness, marked loss of appetite, and marginal declines in body weight (4% of pre-infection weight) and RBC count (7% of pre-infection value). Rectal body temperature increased from a pre-infection mean (± SE) of 38.3 (± 0.2)°C to a high of 38.7 (± 0.2)°C at 7 DPI; however, the increase was not statistically significant (p = 0.06). Trypanosomes were not detected, nor were white cell counts elevated in the CSF (data not shown), confirming that the monkeys were in the first stage of disease when treatment was initiated at 7 DPI. The prodrug DB868 was administered orally to three groups of monkeys: 20 mg/kg/day for 5 days (n = 3), 10 mg/kg/day for 7 days (n = 2), or 3 mg/kg/day for 7 days (n = 2). A fourth group of monkeys (n = 3) was treated intramuscularly with the comparator drug, pentamidine, at 4 mg/kg/day for 7 days (Table 2). Both oral DB868 and intramuscular pentamidine demonstrated efficacy against first stage infection as discussed below. Trypanosome-associated waves of parasitaemaia were not observed (Figure 7), likely because all infections were treated during the first wave of parasitaemia.

DB868 at 20 mg/kg/day x 5 days

Three monkeys, 585, 658 and 686, were treated orally with DB868 at 20 mg/kg/day for 5 days. In all monkeys, trypanosomes were undetectable in blood by direct microscopy or the haematocrit centrifugation technique [24] by the 4th day of drug administration. The monkeys remained trypanosome-free in body fluids (blood and CSF) for the remaining monitoring period (Table 2). Monkeys 686 and 585 were withdrawn from the study and humanely euthanized 4 and 145 days post-LDD, respectively (Table 2), due to the continued deterioration of their health. Trypanosome recrudescence had not occurred in either monkey. Upon post-mortem examination, monkey 686 had peritoneal abscesses while monkey 585 had pneumonia, suggesting that their decline in health was

unrelated to drug therapy. The remaining monkey (monkey 658) was monitored over 525 days post-LDD without any parasitological or clinical evidence of relapse and was declared cured.

DB868 at 10 mg/kg/day x 7 days

Upon treatment with 10 mg/kg/day DB868 orally for 7 days, monkey 691 was parasitologically negative on the 6th day of treatment while monkey 675 became consistently negative 7 days post-LDD. The two monkeys remained negative throughout the remaining post-treatment monitoring period (Table 2). Monkey 675, however, was only monitored up to 114 days post-LDD, at which time it was withdrawn from the study and euthanized due to clinical deterioration. Post-mortem examination indicated pneumonia to be the cause of declining health. Monkey 691 was monitored over 525 days post-LDD without a relapse and was declared cured.

DB868 at 3 mg/kg/day x 7 days

Two monkeys, 638 and 643, were treated orally with DB868 at 3 mg/kg/day for 7 days. Monkey 638 was parasitologically negative on the 6th day of dosing, while monkey 643 was 3 days post-LDD. Both monkeys remained trypanosome-free throughout the extended monitoring period of greater than 500 days and were declared cured (Table 2).

Pentamidine at 4 mg/kg/day x 7 days

Three monkeys, 541, 651 and 672, were administered pentamidine intramuscularly at 4 mg/kg/day for 7 days. Trypanosomes were not detected in monkeys 541 and 651 immediately prior to the third dose. However, trypanosomes were observed intermittently in monkey 672, including 3 days post-LDD when its clinical condition deteriorated, necessitating euthanasia. Monkeys 541 and 651 were monitored over 600 days post-LDD without any evidence of relapse and were declared cured (Table 2).

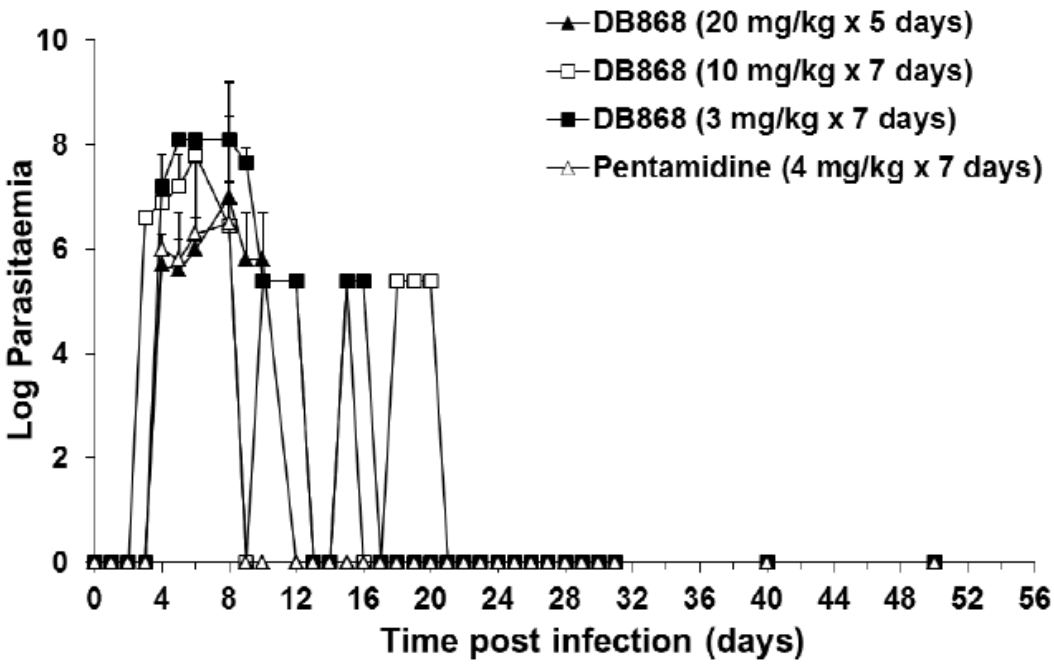

Figure 7. Changes in mean parasitaemia values of vervet monkeys infected with *T. b. rhodesiense* KETRI2537 and subsequently treated with DB868.

Starting at 7 days post-infection, monkeys, confirmed to have first stage HAT, were treated with either DB868 orally or pentamidine intramuscularly. DB868 at 20mg/kg/day for 5 days (n = 2); DB868 at 10 mg/kg/day for 7 days (n = 2); DB868 at 3 mg/kg/day for 7 days (n = 2); pentamidine at 4 mg/kg/day for 7 days (n = 3). Symbols and error bars represent means and interindividual differnces (range), respectively.

Table 2: Efficacy of oral DB868 and intramuscular pentamidine against 1st stage *T. b. rhodesiense* infection in vervet monkeys.

Parameter/Outcome	Oral DB868							Intramuscular Pentamidine		
	20 mg/kg x 5 days			10 mg/kg x 7 days		3mg/kg x 7 days		4 mg/kg x 7 days		
Monkey ID	585	658	686	675	691	638	643	651	541	672
Prepatent period (days post-infection)	5	5	4	5	3	4	4	4	5	6
Peak parasitaemia (Log_{10} P)	7.2	6.9	6.0	7.8	7.8	8.1	8.1	5.4	7.8	5.4
Trypanosomes blood/CSF at EoT	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos
Provisional efficacy at 100 days post-treatment	Cured	Cured	WD	Cured	Cured	Cured	Cured	Cured	Cured	WD
Duration of post-treatment monitoring	145	620	4	114	525	525	525	620	620	2
Final efficacy assesment	WD	Cured	WD	WD	Cured	Cured	Cured	Cured	Cured	Not cured

Key: P = parasitaemia; EoT = end of treatment; WD = withdrawn; Neg = negative; Pos = positive

Pharmacokinetics of DB868 and DB829 in Infected Monkeys

The prodrug DB868 was detected in the plasma of all monkeys with first stage HAT, regardless of the dosing regimen, at 0.04 days (1 h) post-LDD (data not shown). The T_{max} varied between individuals, with the majority (4/6 monkeys) occurring at 0.04 days (1 h) post-LDD. DB868 concentrations declined rapidly. Only one monkey (monkey 675; 10 mg/kg/day for 7 days) had detectable levels at 8 h post-LDD, precluding accurate recovery of pharmacokinetic outcomes for DB868. The median C_{max} for DB829 for the 20 and 10 mg/kg groups (435 and 205 nmol/L, respectively) were 2.6- and 1.2-fold higher, respectively, than that for the 3 mg/kg group (170 nmol/L) (Figure 8; Table 3). The median T_{max} for the 20 and 3 mg/kg groups were similar (4 h), whereas that for the 10 mg/kg group was longer (1 day). The median T_{max} for DB829 in each dose group was longer than that for DB868. The mean AUC_{last} for DB829 in the 20 and 10 mg/kg groups was 10- and 7-fold greater, respectively, than that in the 3 mg/kg group (Table 3). Accurate $AUC_{0-\infty}$ and terminal elimination half-life were only recoverable for the 20 mg/kg group and one monkey in the 10 mg/kg group, precluding between-dose comparisons. The median $AUC_{0-\infty}$ and terminal elimination half-life for the 20 mg/kg group were twice those of the one (monkey 691) in the 10 mg/kg group (Table 3).

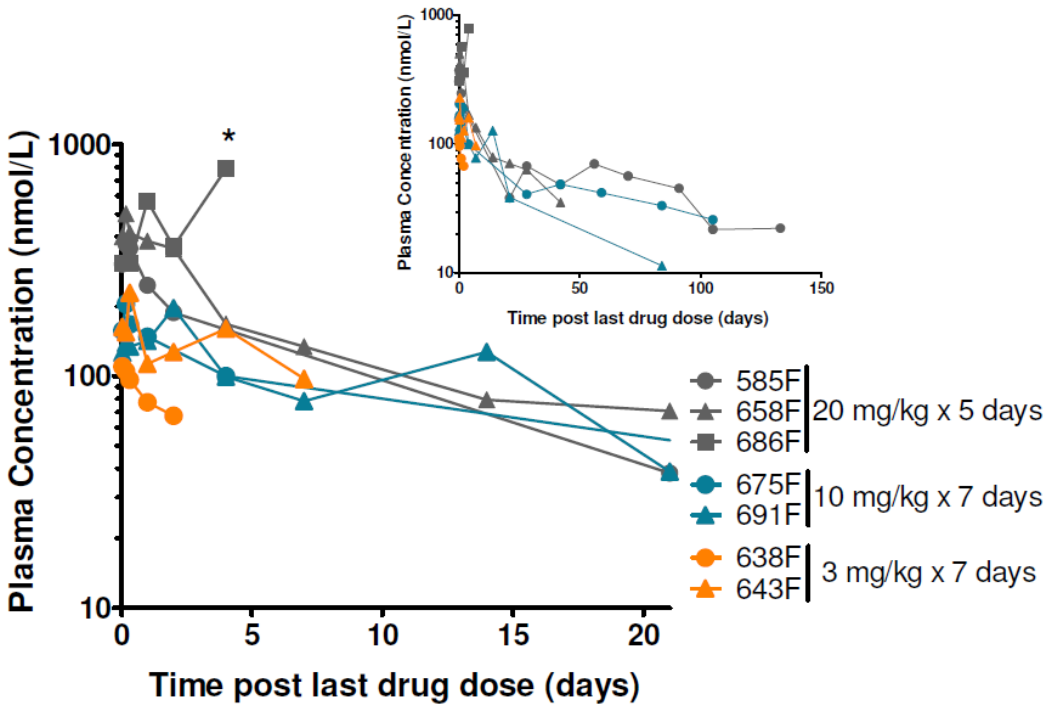

Figure 8. Plasma concentration-time profiles of the active compound DB829 following administration of the prodrug DB868 to vervet monkeys with first stage HAT.

The monkeys were administered DB868 orally, beginning at 7 days post-infection, at 20 mg/kg/day for 5 days, 10 mg/kg/day for 7 days, or 3 mg/kg/day for 7 days, between day -9 to day 0 post-last drug dose. The inset graph shows the extended DB829 profiles up to 150 days post-last DB868 dose. * denotes the time (4 days post-last drug dose) that monkey #686 was euthanized due to clinical morbidity (peritoneal abscesses).

Table 3: Pharmacokinetics of DB829 in vervet monkeys with first stage HAT after the final oral dose of BD868

Outcome	Units	Oral DB868					
		20 mg/kg x 5 days		10 mg/kg x 7 days		3 mg/kg x 7 days	
	Monkey ID	585	658	675	691	638	643
C_{max}	nmol/L	370	500	210	200	110	230
T_{max}	day	0.17	0.17	0.17	2.0	0.04	0.33
AUC_{last}†	nmol/L•day	7300	4100	4000	3500	160	950
$AUC_{0-\infty}$	nmol/L•day	10000	5300	NC	3900	NC	NC
$t_{1/2}$	day	85	24	NC	25	NC	NC

Key: C_{max}, maximum concentration; T_{max}, time to reach maximum concentration; AUC_{last}, area under the curve from time zero to the last measurable concentration; $AUC_{0-\infty}$, area under the curve from time zero to infinite time; $t_{½}$, terminal elimination half-life; NC, not calculable due to >30% extrapolation of the $AUC_{0-\infty}$; † = last measurable concentration varied between monkeys (2-133 days).

DISCUSSION

Consistent with observations involving human liver microsomes [23], vervet monkey liver microsomes metabolized the prodrug DB868 to four intermediate metabolites and the active compound DB829. Similar metabolic pathways have been reported for the related prodrugs pafuramidine and DB844 in rat, monkey and human liver microsomes [20,25-27], demonstrating that these alkoxy-type diamidine prodrugs [28] are converted to active compounds in different animal species. The low DB829 concentrations in the microsomal samples were not unexpected, as similar results were observed with the active compound generated from the related prodrug pafuramidine [17]. The final metabolic steps in the formation of DB829, the *N*-hydroxylation of M2 and subsequently M4, are analogous to those in the conversion of pafuramidine to furamidine. During microsomal pafuramidine metabolism, these steps are catalyzed by cytochrome b_5/b_5 reductase [29]. This enzyme is also abundant in mitochondria and Golgi [29,30], explaining why DB868 is more efficiently converted to DB829 in intact hepatocytes compared to the isolated microsomal system [27,31]. Collectively, these results provided justification for *in vivo* testing of the prodrug DB868 in uninfected and infected vervet monkeys.

No significant overt toxicity was seen with up to 30 mg/kg/day DB868 orally for 10 days (cumulative dose [CD] = 300 mg/kg) in the vervet monkey safety study, suggesting that this dose was below the maximum tolerated dose, but slightly above the no observed adverse effect level (NOAEL). Pharmacokinetic analysis of plasma from the uninfected monkeys showed that the geometric mean C_{4h} of the active compound DB829 in the 30 and 10 mg/kg groups were 23- and 13-fold greater than the IC_{50} (14 nmol/L) against *T. b. rhodesiense* STIB900, respectively. These results confirmed that DB868/DB829 are available systemically following oral administration, similar to pafuramidine/furamidine and DB844/DB820 [20,32], prompting further evaluation in the monkey model of first stage HAT.

Based on the above observations, DB868 efficacy was evaluated in vervet monkeys with first stage HAT using doses below 30 mg/kg/day in order to minimise the

risk of unfavourable clinical outcomes. Dosing durations of 5-7 days were chosen based on the hypothesis that first stage disease can be cured using short treatment durations. DB868, administered orally, cured all monkeys of their experimentally introduced *T. b. rhodesiense* infection (Table 2). Post-treatment monitoring must be at least 180 days in order to declare a cure in the monkey model of first stage HAT [16]. All three DB868 dosing regimens, including the lowest evaluated (3 mg/kg/day for 7 days; CD = 21 mg/kg), effectively cleared the monkeys of their considerably high parasitaemia, which in some cases was as high as 10^8 trypanosomes/mL of blood. Elimination of the pathogens allowed the monkeys to return to their clinical and haematological baselines within one month post-LDD (data not shown), similar to what was observed in pafuramidine and DB844 efficacy studies conducted in this monkey model [16,20]. These results highlight the ability of diamidines to eliminate injurious trypanosomes, allowing the body to repair/heal itself. Furthermore, oral DB868 appears to be superior to oral pafuramidine in this first stage HAT monkey model, as a higher dose of pafuramidine than DB868 was required to achieve complete cure (10 mg/kg for 5 days *vs.* 3 mg/kg for 7 days, respectively) [16]. The longer dose regimen for DB868 compared to pafuramidine is consistent with the *in vitro* observation that DB829 required longer exposure periods than furamidine to kill *T. b. brucei* s427 trypanosomes [33]. For example, a 24-h exposure to DB829 (2.7 μM) was required, whereas a 1-h exposure to furamidine (3.2 μM) was required to kill these trypanosomes in culture.

Based on a mg dose basis, DB868 has a larger therapeutic window than pafuramidine in vervet monkeys. No notable drug-induced overt toxicity was observed in either uninfected or infected monkeys administered DB868, except for mild excess mucous in the stool (n = 1) and transient inappetance (n = 2) in the group receiving 30 mg/kg/day for 10 days. In comparison, similar mild adverse events were observed when pafuramidine was administered at 10 mg/kg/day for 10 days to vervet monkeys (unpublished data; JK Thuita). DB868, at all doses tested, did not cause significant elevations in plasma biomarkers of liver (ALT, AST, total and direct bilirubin; Figure 4) and kidney (creatinine; Figure 5) injury. These results contrasted with those of pafuramidine, which caused transient liver injury during an extended phase I clinical trial

in humans [11]. In addition, only a slight increase in ALT (less than 2-fold) was observed in female Sprague-Dawley rats administered DB868 orally (25 mg/kg/day for 3 weeks) compared to untreated rats, whereas an 18-fold increase was observed in rats administered pafuramidine (12 mg/kg/day for 4 weeks) [34]. In the current study, plasma urea concentrations, and therefore BUN levels, were transiently increased (2-3-fold; Figure 5B) shortly (1-2 days) after the last drug dose. However, the BUN:creatinine ratio was above the critical 20:1 ratio, suggesting that the elevations were likely due to pre-renal causes such as dehydration. Direct comparison of plasma liver and kidney injury biomarkers between DB868 and pafuramidine in vervet monkeys are not possible due to insufficient data on pafuramidine. Nevertheless, the safety profile of DB868 is improved over that of the prodrug DB844, which caused significant liver injury when administered to monkeys at doses above 10 mg/kg/day [20], necessitating withdrawal of DB844 from further development.

As discussed above, our study has demonstrated that oral DB868 has excellent efficacy and an improved therapeutic window in the first stage HAT monkey model, making it a promising lead candidate for further preclinical development. However, based on the previous lessons learned from the development of pafuramidine [11], several issues warrant mention. First, the kidney safety liability of DB868 needs to be further examined using more predictive models and biomarkers. Pafuramidine development was terminated due to an unexpected severe kidney injury that occurred in five patients (~6%), a liability not predicted by traditional preclinical safety testing in rodents [11]. Recently, Harrill *et al*. [21] showed, using a mouse diversity panel comprised of 34 genetically diverse inbred mouse strains, marked elevations of urinary kidney injury molecule-1 (KIM-1) in sensitive mouse strains following oral administration of pafuramidine, while classical kidney injury biomarkers, BUN and serum creatinine, remained unchanged. Hence, it may be prudent to screen DB868 for kidney injury liability using the sensitive mouse strains therein identified and KIM-1. Encouraging results from Sprague Dawley rats administered pafuramidine or DB868 orally (12 mg/kg/day x 28 days) showed that DB868 had no effect on KIM-1 during the entire 4-month observation period (28 days of drug administration and 92 days of

recovery period), whereas pafuramidine caused a 13-fold increase in KIM-1 one week post-LDD [34].

Second, treatment regimens should be optimized with the pharmacokinetics taken into consideration. The active compound DB829 was readily detected in the plasma following oral administration of the prodrug DB868 (Figures 6 and 8). Afterwards, DB829 was slowly eliminated from the blood with a terminal elimination half-life ranging from days to nearly three months depending on the dosing regimen (Tables 1 and 3). This is similar to suramin [7], the only other first stage HAT drug besides pentamidine. Plasma concentrations of DB829 remained >100 nmol/L for long periods following the last DB868 dose, in some monkeys up to 7 days post-LDD. This finding was comparable to that reported for DB844 [20] and provides additional evidence that 1) prolonged treatment durations may not be necessary, especially for first stage HAT, and 2) daily dosing of DB868 and other diamidine prodrugs may not be necessary. However, since trypanosomes are tissue invasive, a follow-up pharmacokinetic study is needed to determine if plasma active drug concentrations are predictive of tissue concentrations.

Third, combined treatment of DB868 with a fast-acting trypanocide may accelerate recovery, improve efficacy and clinical outcomes, and prevent resistance. The time to clearance of trypanosomes from peripheral blood was shorter in monkeys treated with pentamidine intramuscularly (2 days after the 1st 4 mg/kg dose) than with oral DB868 (2-14 days after the 1st dose depending on the dose; Figure 7). It took longer for the lower DB868 dose regimen groups (3 and 10 mg/kg) to clear parasites from the blood than the 30 mg/kg group (6-14 days *vs.* 2-5 days after the 1st drug dose; Figure 7). The difference in parasite clearance between pentamidine and DB868 (or the active compound DB829) is consistent with observations in mouse models of HAT (Wenzler *et al.*, *Antimicrob Agents Chemother.* under review). However, the slower parasite clearance by DB868 did not seem to compromise efficacy in the monkey model. Nevertheless, combining oral DB868 with another fast-acting trypanocidal agent, such as the oral drugs currently in clinical trials, may offer fast elimination of parasitaemia, the ease of oral pills, and a low probability of developing resistance.

In conclusion, oral DB868 demonstrated improved efficacy and safety profiles in the vervet monkey model of first stage HAT, in comparison to the previous clinical candidate pafuramidine. As such, DB868 should be considered a preclinical candidate for oral treatment of first stage HAT, supplementing the current drug development pipeline.

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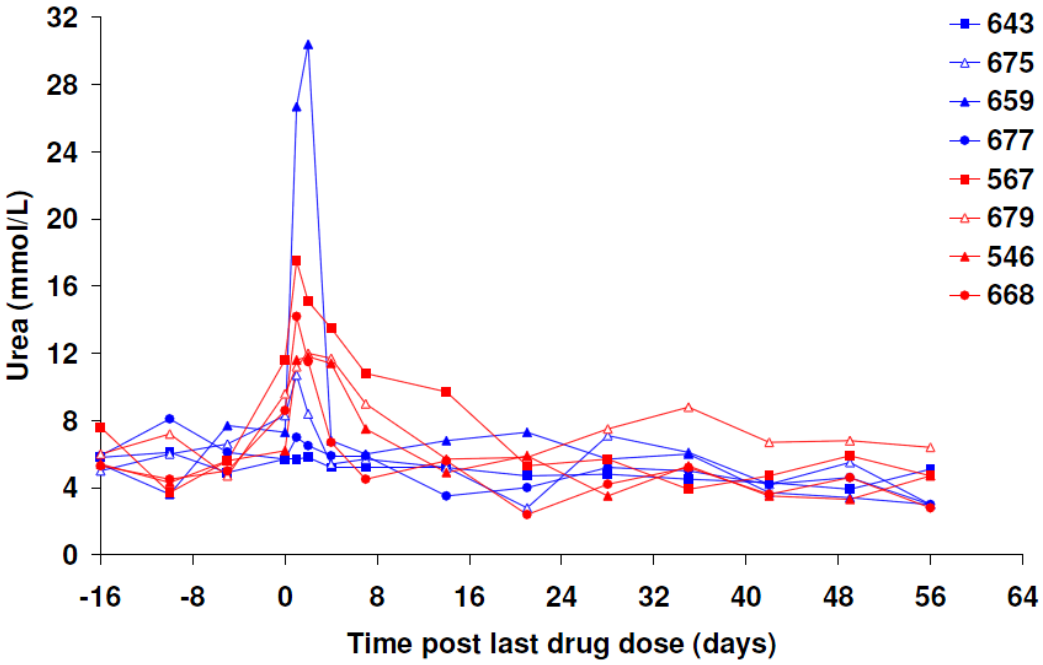

Figure S1: Individual plasma urea concentration-time profiles of un-infected vervet monkeys administered DB868. DB868 was administered orally at 10 mg/kg/day (643, 675, 659, 677; blue symbols) or 30 mg/kg/day (567, 679, 546, 668; red symbols) for 10 days between day -9 to day 0 post-last drug dose.

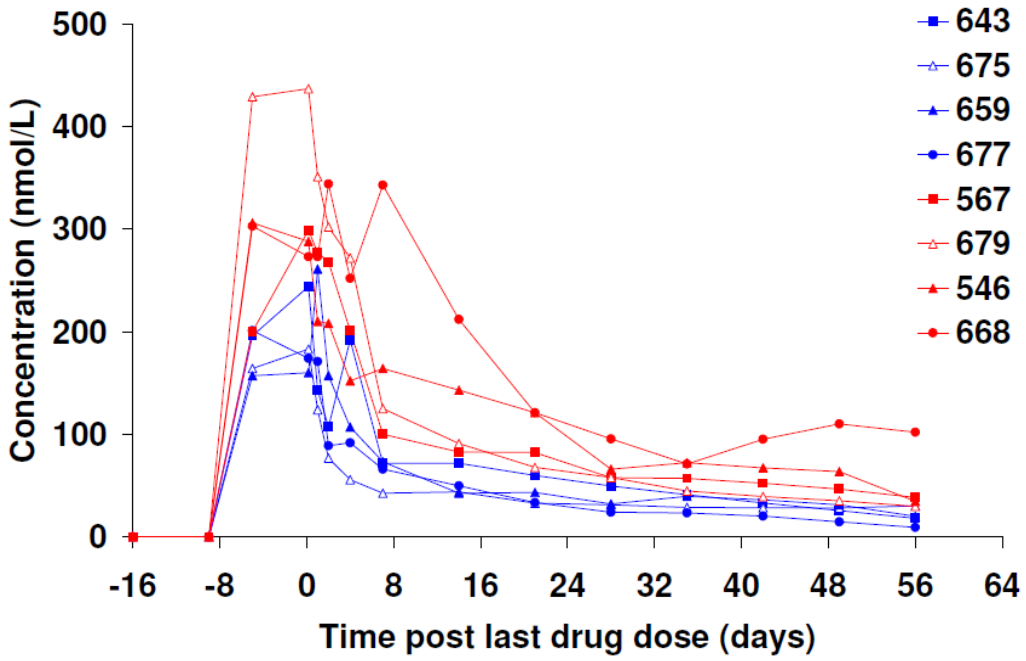

Figure S2: Individual plasma concentration-time profiles of the active compound DB829 following administration of DB868 to un-infected vervet monkeys. DB868 was administered orally at 10 mg/kg/day (643, 675, 659, 677; blue symbols) or 30 mg/kg/day (567, 679, 546, 668; red symbols) for 10 days between day -9 to day 0 post-last drug dose.

Chapter 7: Chemotherapy of Second Stage Human African Trypanosomiasis with Parent Diamidines or their Oral Prodrugs: which way to go?

[1,7]*John K Thuita, [2]Kristina K Wolf, [1]Grace A Murilla, [1]James N Mutuku, [3]Susan K Jones, [4]David W Boykin, [3]Richard R Tidwell, [2]Mary F Paine, [5]Michael Z Wang and [6,7]Reto Brun

[1]Trypanosomiasis Research Centre, Kenya Agricultural Research Institute (TRC-KARI) P.O box 362-00902, Kikuyu, Kenya.
[2]Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, North Carolina, U.S.A
[3]Pathology Department, School of Medicine, University of North Carolina at Chapel Hill, North Carolina, U.S.A
[4]Department of Chemistry, Georgia State University, Atlanta, Georgia, U.S.A.
[5]Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas, U.S.A
[6]Swiss Tropical and Public Health Institute, Socinstrasse 57, CH-4002 Basel, Switzerland
[7]University of Basel, Petersplatz 1, CH-4003 Basel, Switzerland
*Corresponding author: e-mail address: thuitajk@yahoo.com

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ABSTRACT

Human African trypanosomiasis (HAT, sleeping sickness) ranks among the most neglected tropical diseases (NTDs), based on limited availability of safe and efficacious drugs especially for the late central nervous system (CNS) stage disease. Since approximately year 2000, considerable efforts have been made to redress this situation mainly by not-for-profit product development partnerships (PDPs) such as Drugs for Neglected Diseases initiative (DNDi) and the Consortium for Parasitic Drugs Development (CPDD). The CPDD in particular has developed novel parenterally administered diamidines and orally administered diamidine prodrugs which have shown potential for treatment of CNS stage HAT in murine studies. To provide a rationale for selection of either parenteral or orally administered compounds for further development, the pharmacokinetics and efficacy of intramuscularly (IM) administered active drug 2,5-bis[5-amidino-2pyridyl]furan (DB829, CPD-0802) and its orally administered prodrug 2,5-bis [5-(*N*-methoxyamidino)-2-pyridyl] furan (DB868) were investigated in the vervet monkey model of CNS stage HAT. Treatments with either compound were initiated 28 days post infection of monkeys with *T. b. rhodesiense* KETRI 2537. Results showed that IM DB829 at either 5 or 2.5 mg/kg/day for 5 days or as five doses of 5 mg/kg/day administered on alternate days cured 2/2 monkeys at each dose level. The prodrug (DB868) was less successful, with a cure rate of 1/4 at either 20 or 10 mg/kg/day x 10 days per os and no cures at 3 mg/kg/day x 10 days. Pharmacokinetic analysis demonstrated that at 5 mg/kg of IM DB829, the plasma C_{max} values were 25 times greater than DB829 C_{max} values obtained after oral dosing of DB868 at 20mg/kg, suggesting that enhanced systemic availability of the active drug was the reason for better efficacy seen with IM dosing. These data show that the active diamidine DB829 should be considered for further development as a potential new treatment for CNS stage HAT.

Key words: Novel diamidines, CNS stage HAT, chemotherapy, vervet monkeys, Pharmacokinetics

Short title: Chemotherapy of CNS-stage HAT with parenteral or oral diamidines

INTRODUCTION

Diamidines are widely used in the control of infectious diseases of both man and animals. In humans, pentamidine is used to treat first stage sleeping sickness (human African trypanosomiasis, HAT), leishmaniasis and *Pneumocystis jirovecii (carinii)* pneumonia which occurs mostly in AIDS patients (Tidwell *et al*., 1990). Diminazene aceturate, a diamidine that is primarily used in livestock medicine, has demonstrated activity against *Babesia (bovis* and *bigemina*) and a variety of animal infective trypanosomes. The development of the diamidines class of compounds was made possible by breakthrough discoveries that pathogenic trypanosomes required large amounts of sugar for their metabolism, indicating that their pathogenicity could be modulated by lowering plasma glucose levels (Sterverding, 2010). This was followed closely by findings that synthalin, a hypoglycaemic drug, exhibited antitrypanosomal activity in rodents that was independent of its ability to lower blood glucose levels (Bray *et al*., 2003; Steverding, 2010). The first aliphatic diamidines were made through structure-activity relationship studies (SARs) of the synthalin scaffold.

Despite the proven utility of the aliphatic diamidine, pentamidine, as a therapeutic agent for first stage *T. b. gambiense* HAT (WHO, 2012), and the synthesis of aromatic diamidines such as furamidine (DB75) (Das and Boykin, 1977; Boykin *et al*, 1996), no new diamidines have entered clinical use for HAT or any other human diseases. Since the year 2000, however, there has been renewed research and development (R&D) focus on the diamidines with the objective/s of developing compounds with: i) good systemic bioavailability after oral administration since such compounds could be more readily used in the resource poor settings where HAT was common, ii) good activity against the difficult to treat second stage HAT that is characterised by trypanosome invasion of the cerebrospinal fluid (CSF), brain and associated membranes (meninges), causing menigoencephalitis (Kennedy, 2004). As a first step to address these objectives, novel diamidine prodrugs in which the positive charges on diamidines were masked by alkoxy molecules were developed (Ismail *et al*., 2003). The new prodrugs, including DB289 and DB844, were subsequently shown to have improved transport across Caco-2 cell monolayers in comparison with their parent diamidines, DB75 and DB820 respectively

(Zhou *et al*., 2002; Ansede *et al*., 2004). They were also fully curative in mouse models of first and second stage HAT after oral administration (Boykin *et al*., 1996; Ansede *et al*., 2004; Thuita *et al*., 2008; Wenzler *et al*., 2009). In monkeys, pafuramidine (DB289) was shown to be fully curative against first stage HAT (Mdachi *et al*., 2009) thus providing additional evidence that the prodrug strategy was effective in delivering diamidines across biologic membranes in different animal species. However, efficacy of the prodrugs against second stage HAT in monkeys ranged from a poor 0% for oral pafuramidine (Mdachi *et al*., 2009) to a moderately improved 43% for oral DB844 (Thuita *et al*., 2012), suggesting the need to rethink the strategy to identifying potential therapies for second stage HAT.

An alternative pair of active drug, 2,5-bis[5-amidino-2pyridyl]furan (DB829) and its orally administered prodrug 2,5-bis [5-(*N*-methoxyamidino)-2-pyridyl]furan (DB868), were also shown to be fully curative in the second stage GVR35 mouse model of HAT (Wenzler *et al*., 2009), suggesting that both molecules had good blood brain barrier (BBB) permeability in this species . On the basis of these data, DB868 progressed to the monkey model where we demonstrated that it was well tolerated in un-infected monkeys and was fully curative in the first stage vervet monkey model of HAT (Thuita *et al*., PLoS NTDs, in press). However, the activity of orally administered DB868 and parenterally administered DB829 against central nervous system (CNS) stage HAT in monkeys remained undetermined. In the current study, we therefore investigated the efficacy and pharmacokinetics of both DB829 after intramuscular (IM) administration and DB868 after oral administration in monkeys in order to determine which of the two test compounds would be better suited for treatment of second stage HAT. In addition, monkeys that relapsed after treatment with the diamidines were identified and retreated with the organo-arsenic drug melarsoprol to determine the continued sensitivity of the isolate (*T.b. rhodesiense* KETRI2537) to the recommended treatment for CNS stage eastern African HAT (WHO, 2012). Finally, monkeys were evaluated for time to relapse characteristics, haematological parameters and cerebrospinal fluid (CSF) white cell counts to gain insights on their utility as biomarkers of disease progression, resolution of disease post trypanocidal therapy and/or early detection of relapse in this model.

MATERIALS AND METHODS

Ethics

Studies were undertaken in adherence to experimental guidelines and procedures approved by the Institutional Animal Care and Use Committee (IACUC), the ethical review committee for the use of laboratory animals at the Trypanosomiasis Research Centre of the Kenya Agricultural Research Institute (TRC-KARI). The experimental guidelines also complied with National guidelines set by the Kenya Veterinary Association.

Trypanocidal Drugs

The test articles were two diamidines (DB868 and DB829) which were originally synthesised in the laboratories of Dr David W Boykin (Ismail *et al.*, 2003). The drugs were presented in the form of yellow powder in opaque and water tight bottles.

a) 2,5-bis[5-amidino-2pyridyl]furan (DB829 [CPD-0802] Lot # SP117-ACE-P5, MW = 451.648) which was synthesised by Solvias AG, Basel, Switzerland) and had a chemical purity of 97.2% as determined by ^{1}H NMR and HPLC (Susan Jones, personal communication). In the laboratory, DB829 was reconstituted in 5% dextrose (D5W) to render concentrations of 10 mg/ml and 5 mg/ml and administered to monkeys at 0.5 ml/kg intramuscularly (IM) as recommended (http://www.findthatpdf.com/search-19180523-hPDF/download-documents-volume_guidelines.pdf.htm). Each dose was divided into two aliquots and administered at two sites on the same limb while the next day's dose was administered on the alternate limb. Dosing formulations were prepared daily and protected from light (using aluminium foil) between preparation and actual injection into the monkeys which took a maximum of 30 minutes.

b) 2,5-bis [5-(*N*-methoxyamidino)-2-pyridyl] furan (DB868, Lot # 2-JXS-28, MW = 564.370) which was synthesised by Scynexis Inc., Research Triangle Park, NC, USA). It had a chemical purity of 95.6 as determined by NMR and High performance liquid chromatography (HPLC). In the laboratory, DB868 was wrapped in aluminium foil and stored at room temperature (23-25°C). Dosing

solutions were prepared daily by reconstituting the drug in distilled de-ionised water in three concentrations of 10, 5, and 1.5 mg/ml and administered to monkeys per os (PO) at 2 ml/kg body weight.

c) Melarsoprol (Mel B, Arsobal), was supplied ready to use by the WHO in glass ampoules as a 3.6% solution in propylene glycol. It was administered to the monkeys intravenously (IV) at 3.6 mg/kg body weight for four days.

Trypanosomes

The isolate used in this study was *T. b rhodesiense* KETRI 2537 which was isolated in 1989 and cryopreserved in the TRC-KARI trypanosome bank as previously described (Fink and Schmidt, 1980).

Experimental Animals

A total of eighteen (18) vervet monkeys [*Chlorocebus(Cercopithecus) aethiops* , syn. African green monkeys] weighing between 2.5 and 5.5kg that were already in the colony maintained at TRC-KARI were used. These monkeys had initially been acquired from the Institute of Primate Research (IPR) in Kenya and had been subjected to the routine 90-day quarantine procedures that are designed to ensure their freedom from infectious diseases, especially the zoonotics (Gichuki and Brun, 1999; Thuita *et al*, 2008b). The monkeys were housed in stainless steel cages and were fed a diet of fresh vegetables and commercial monkey cubes (Unga feeds, Nakuru Kenya) twice daily and given water *ad libitum* (Thuita *et al*, 2008b).

Study Design

The efficacies of the two test articles, orally administered prodrug DB868 and intramuscularly (IM) administered active drug DB829 (CPD-0802) were evaluated in two separate experiments. In each experiment, baseline data (weight, clinical and haematology parameters) were collected for 2 weeks after which monkeys were infected by intravenous injection of approximately 10^4 *T. b. rhodesiense* KETRI 2537

trypanosomes diluted from infected blood of immuno-suppressed donor Swiss white mice (Thuita *et al*., 2012). The matching method of Herbert and Lumsden (1976) and the haematocrit centrifugation technique (HCT) (Woo, 1970) were used to collect parasitaemia data. Therapeutic intervention was initiated at 28 DPI after establishing that the animals had second stage disease using the presence of trypanosomes in CSF and elevated white cells (> 5 cells/µl) in CSF as biomarkers (WHO, 2012). CSF was processed as previously described (Miezan *et al.*, 2000; Thuita *et al*., 2012).

As a result of a regulator instituted freeze on acquisition of new non-human primates, only six monkeys were available for the IM DB829 study. These were divided into three treatment groups, each group consisting of two monkeys (one male and one female), to give a preliminary indication of dose response and compare consecutive-day with alternate-day dosing. They were treated at 5 mg/kg x 5 days consecutively (monkeys 569 and 659), 5 mg/kg administered every alternate day for 5 days (monkeys 668 and 676) and 2.5 mg/kg x 5 days consecutively (monkeys 546 and 693) (Table 1).

Another 12monkeys were available for the efficacy study of oral DB868. These monkeys were divided into treatment groups, each consisting of two male and two female monkeys, for oral treatment with DB868 at 20 mg/kg x 10 days consecutively (monkeys 573, 679, 689 and 696) and 10 mg/kg x 10 days consecutively (monkeys 688, 690, 695 and 697). A third treatment group, 3 mg/kg x 10 days consecutively, contained only two monkeys (numbers 670 and 687) (Table 1) since two other monkeys which had been allocated to this group did not become blood or CSF trypanosome positive at all and were consequently withdrawn from the study.

Post treatment monitoring for clinical and parasitological cure as well as for haematologic and pharmacokinetic parameters, were carried out as previously described (Thuita *et al*., 2012). To facilitate collection of blood and CSF for these assessments, the monkeys were anaesthetised with ketamine HCl at 10-15 mg/kg and valium 0.5 mg/kg.

HPLC-MS/MS Quantification of DB868 and DB829 in Monkey Plasma

Monkey plasma samples were processed for quantification of DB829, DB868 or pentamidine using modifications of methods previously described in Harrill *et al*. (2012)

and Wang *et al*., 2010; the resultant modified methods used to assay monkey biological fluids for DB829/DB868 concentrations has been described in detail (Thuita *et al*., PLoS NTDs, in press).

Data Analysis

All clinical and haematological data were entered and managed using Microsoft Excel (Version 2003). The data were analysed using Statview for Windows Version 5.0.1 (SAS Institute Inc, 1995–1998, Cary, NC). Repeated measures ANOVA was performed to test the effect of trypanosome infection on haematology and clinical chemistry parameters as well as the effect of IM DB829 and oral DB868 on the same parameters ($\alpha = 0.05$). In addition, 95% confidence intervals were derived to further test the significance of observed findings. The PK data were analysed using the non-compartmental analysis (NCA) with the aid of the WinNolin programme.

RESULTS

Parasitaemia, CSF Parasitosis and Clinical Disease before Treatment

Following intravenous injection of approximately 10^4 trypanosomes per monkey, median (range) pre-patent periods of 4.5 (4-7) and 4 (3-5) days were obtained for the monkeys in the efficacy studies of oral DB868 and IM DB829, respectively (Table 1). Thereafter, the parasitaemias were characterised by average peak values of 10^7 trypanosomes/mL of blood (Table 1), with the characteristic fluctuations associated with trypanosome infections (Figures 1 and 2); these observations were similar to primary parasitaemia data previously reported for this model (Thuita *et al.*, 2012). Clinically, the first stage disease was characterised by transient inappetance, rough haircoats, fluctuating fever and weight loss (< 5%). General lymphadenopathy and enlargement of the spleens (up to 3 times pre-infection sizes) were also observed.

Cerebrospinal fluid (CSF) parasitosis was confirmed in all the monkeys before the last pre-treatment sampling point at 27 DPI (Table 1). The trypanosome densities were low at 1-2/μL of CSF (Table 1); in a majority of the monkeys, the parasites were however detected on more than one occasion during the weekly physical examination and sample collection activities before initiation of treatment (data not shown). In addition, white cell numbers in CSF were modestly elevated in comparison with baseline values as shown (Figure 3), thus confirming that the monkeys were in the central nervous system (CNS) stage disease (WHO, 1998; WHO, 2012). However, only a limited number of monkeys exhibited some clinical signs of late stage disease, including hind limb paresis, altered behaviour and chirping. They were treated with different dose regimens of either DB868 or DB829 from 28 days post infection (DPI).

Efficacy of DB829 Dose Regimens

DB829 was administered intramuscularly (IM) at 5 mg/kg for 5 days consecutively (monkeys 569 and 659) or at 5 mg/kg every alternate day for 5 days (monkeys 668 and 676). A third group of monkeys was treated at 2.5 mg/kg for 5 days consecutively (Table 1). Monkeys that were treated using either of the 5 mg/kg dose regimens became blood and CSF parasite negative by the last day of dosing. In contrast,

trypanosomes were more persistent in the biological fluids of monkeys that were dosed at 2.5 mg/kg; the trypanosomes were still detectable in blood and/or CSF for 4 days (monkey 693) and 7 days (monkey 546) post last dosing. Even in these two monkeys (546 and 693) however, trypanosomes were thereafter not detected in biological fluids, indicating that all the monkeys that were treated with DB829 were parasite negative at the last time point (14 days post last dosing) that could still be considered an end of treatment (EoT) evaluation (Table 1). All the monkeys subsequently regained their normal clinical condition. Five of the monkeys (569, 668, 676, 546 and 693) remained free of parasites in blood and CSF during the more than 300 days of post treatment monitoring (Table 1) and were therefore declared cured. However, monkey 659 of the 5 mg/kg x 5 days consecutive daily dosing regimen, developed pneumonia and had to be euthanized 91 days post last dosing. Trypanosome recrudescence was not observed in blood or CSF collected from this monkey at any time point post last dosing. The monkey (659) was therefore classified as withdrawn from the study (Table 1).

Efficacy of Oral DB868 Regimens

The prodrug DB868 was dosed orally to groups of four monkeys using 10-day dose regimens of 20, 10 and 3 mg/kg, respectively. Monkeys in the 20 and 10 mg/kg dose-groups became blood and CSF parasite negative by the 7^{th} day of dosing and all were therefore provisionally cured at the end of treatment (EoT) examination (Table 1). The two monkeys that were treated at 3 mg/kg (No 670 and 687) also experienced aparasitaemia after the 7^{th} drug dose. However, the central nervous system (CNS) trypanosomes were not eliminated at all, indicating that the 3 mg/kg x 10 days regimen did not achieve cure (Table 1).

Three of the four monkeys that were treated orally with DB868 at 20 mg/kg experienced trypanosome recrudescence at various times post last drug dose (LDD), giving a final cure of 1/4 for this group (Table 1). All three animals (monkeys 573, 689 and 696) were confirmed to have CSF trypanosomes, CSF white cell aberrations and/or clinical signs of CNS disease (data not shown). However, only one, monkey 689, experienced trypanosome recrudescence in the blood (Figure 2). The median (range) time

to relapse was 133 (77-161) days. Clinical signs of CNS involvement included ataxia, circling and altered behaviour and were quite pronounced in monkey 696, necessitating humane euthanasia of the monkey. The remaining two monkeys (573 and 689) were successfully rescue-treated with melarsoprol (Mel B, Arsobal®) at 3.6 mg/kg x 4 days intravenously.

Similarly, three out of the four monkeys that were treated with DB868 at 10 mg/kg relapsed. The three monkeys (Monkey 688, 695 and 697) were all positive for blood trypanosomes (Figure 2) and two of them (688 and 697) also were positive for CSF trypanosomes and/or clinico-pathological indicators of late CNS stage disease. The median (range) time to relapse was 28 (28-56) days. In these monkeys, relapse parasitaemia hovered around the limit of detection of Herbert and Lumsden (1976) and tended to be of a lower intensity in comparison with primary parasitaemia in the same monkeys (Figure 2). In contrast, CSF white cell aberrations were more pronounced in the relapsed monkeys, in comparison with the period before treatment (Figure 3 and data not shown). The three monkeys were also successfully rescue-treated with melarsoprol (Mel B, Arsobal®) at 3.6 mg/kg x 4 days intravenously.

In all cases when monkeys were retreated with melarsoprol, trypanosomes were cleared from the peripheral blood by the second day of treatment confirming that melarsoprol is a fast acting trypanocide. The post treatment monitoring period was restarted after the last dose of melarsoprol and continued for at least 300 days before cure was declared. The monkeys remained free of trypanosomes and had normal clinical and haematology parameters; they were therefore confirmed cured. Monkey 687 was however not cured; its clinical condition deteriorated significantly after two daily doses of melarsoprol treatment, necessitating it to be euthanized.

Table 1: Efficacy of intramuscular DB829 and oral DB868 dose regimens in a second stage monkey model of HAT

Experiment	An #	PP (DPI)	Peak $Log_{10}P$	Time to parasitisation of CSF (DPI)	# of tryps/ µl of CSF	Dose regimen (mg/kg x # of days	Time of treatment (DPI)	Efficacy at EoT	ToC at 300 DPI	Cured/ treated
I: IM DB829	569	4	7.2	27	1	5 x 5	28-32	Neg	Cured	1/1
	659	4	7.8	27	1			Neg	WD (91)	
	668	5	7.8	21	1	5 x 5	28, 30, 32, 34, 36	Neg	Cured	2/2
	676	5	7.5	21	1			Neg	Cured	
	546	4	7.5	27	1	2.5 x 5	28-32	Neg	Cured	2/2
	693	3	8.1	14	1			Neg	Cured	
Average		4 (3-5)	7.7 ± 0.1	24 (14-21)	1	NA	NA	NA	NA	
I: Oral DB868	573	4	8.1	14	2	20 x 10	28-37	Neg	Relapsed	1/4
	679	5	7.8	27	1			Neg	Cured	
	689	5	7.8	27	1			Neg	Relapsed	
	696	4	7.8	7	1			Neg	Relapsed	
	688	5	7.5	21	1	10 x 10	28-37	Neg	Relapsed	1/4
	690	5	7.8	14	1			Neg	Cured	
	695	4	7.5	7	1			Neg	Relapsed	
	697	4	7.8	7	1			Neg	Relapsed	
	670	7	7.2	NA	NA	3 x 10	28-37	Neg	WD	
	687	4	7.8	21	2			Neg	Not cured	0/2
Average		4.5 (4-7)	7.8 ± 0.1	10.5 (7-27)	1 (1-2)	N/A	N/A	N/A		

An #, animal number; PP, pre-patent period; DPI, days post infection; P, parasitaemia; IM, intramuscular; EoT, end of treatment; tryps, trypanosomes; CSF, Cerebrospinal fluid; WD, withdrawn; Neg, negative; N/A, not applicable; ND, not demonstrated; ToC, test of cure peforemd after 300 days post LDD

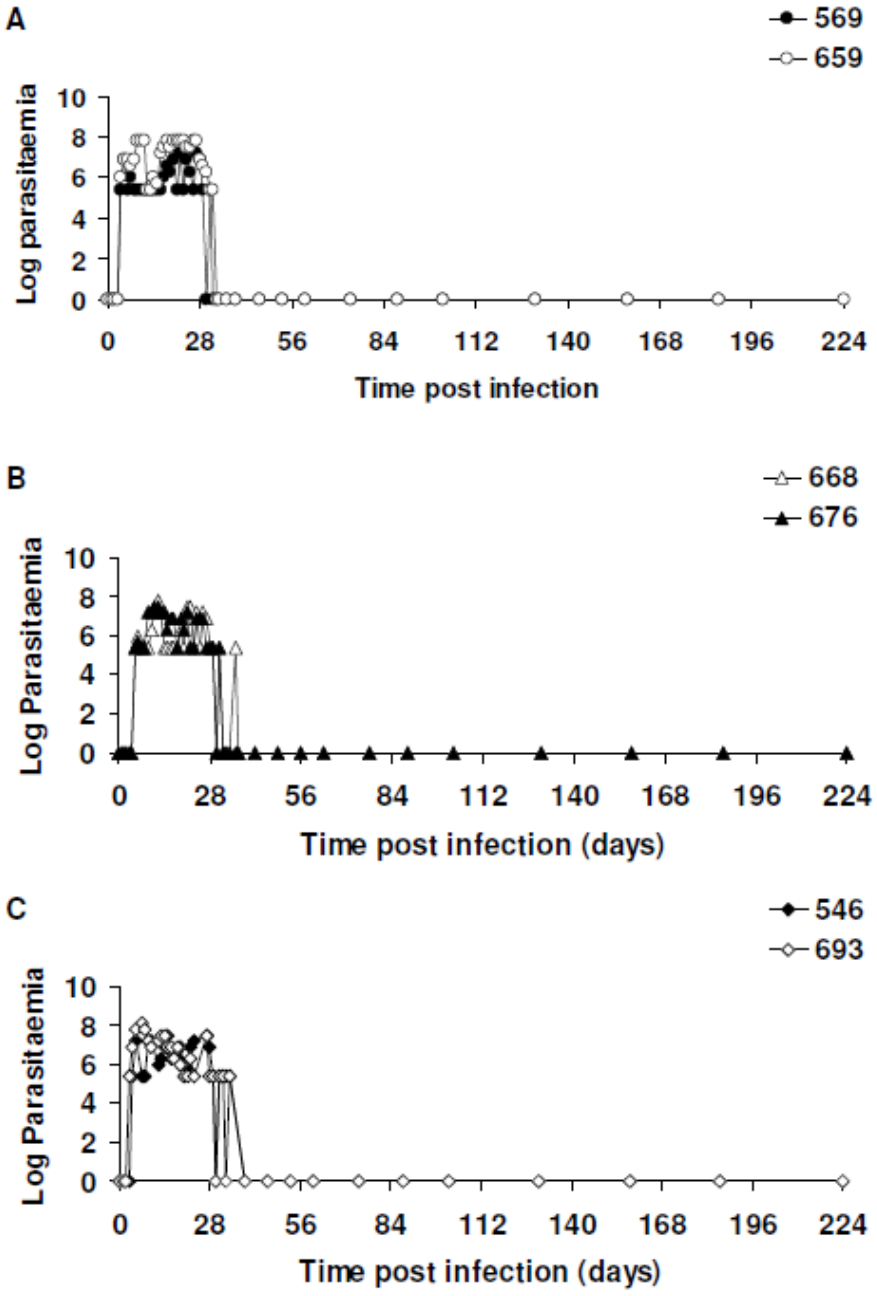

Figure 1: Parasitaemia progression in monkeys infected with *T. b. rhodesiense* KETRI 2537 and later treated with DB829. DB829 was administered intramuscularly from 28 days post infection at: - A: 5 mg/kg x 5 days consecutively; B: 5 mg/kg every alternate day; C: 2.5 mg/kg x 5 days consecutively.

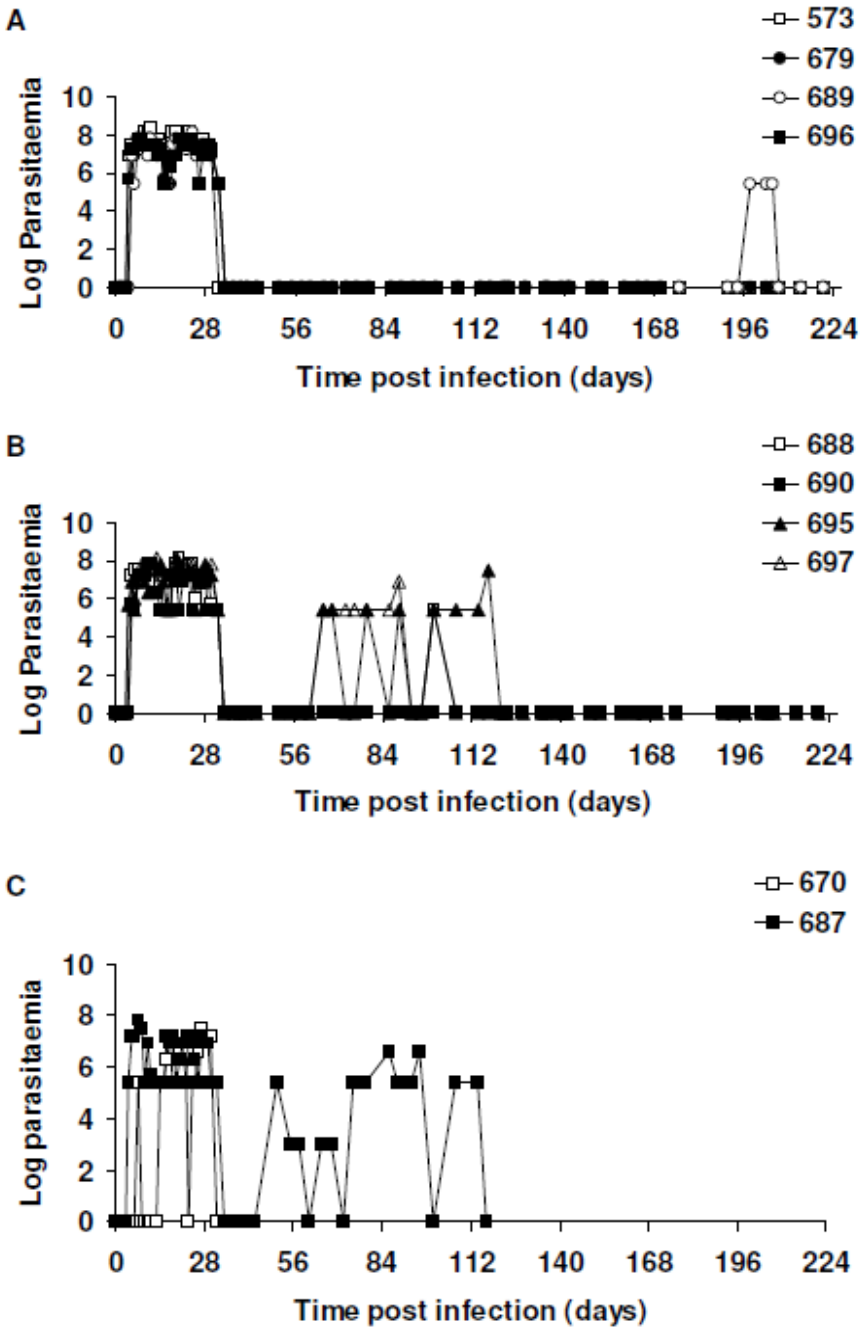

Figure 2: Primary and relapse parasitaemia in monkeys infected with *T. b. rhodesiense* KETRI 2537 and subsequently treated with prodrug DB868. DB868 was administered orally from 28 days post infection at: - A: 20 mg/kg x 10 days consecutively; B: 10 mg/kg x 10 days consecutively; C: 3 mg/kg x 10 days consecutively.

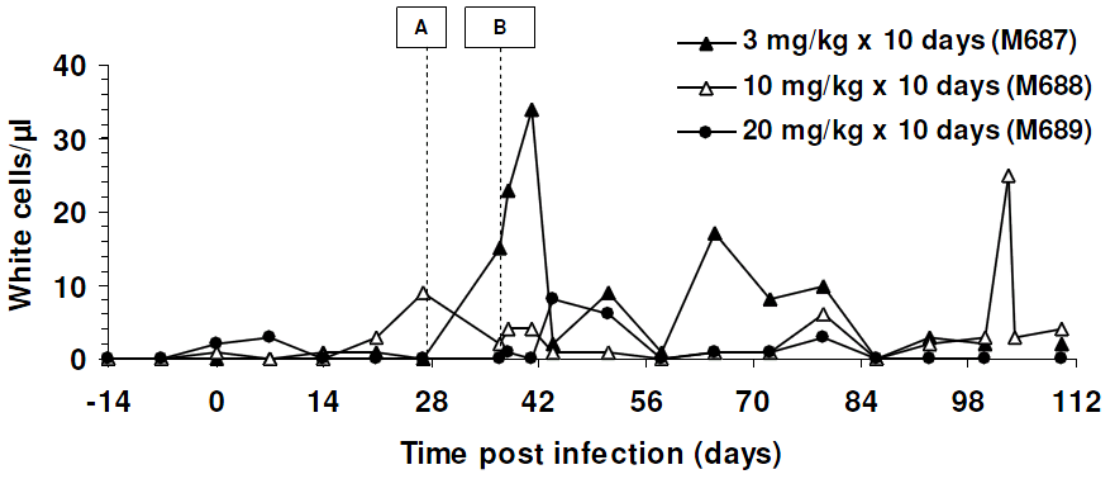

Figure 3: Cerebrospinal fluid white cell changes in monkeys that were treated with oral DB868 regimens. The monkeys were dosed from 28 (A) to 37 (B) days post infection with *T.b. rhodesiense* KETRI 2537.

Infection and Treatment Induced Changes on Haematology

Red blood cell (RBC) counts exhibited an infection-induced decline that was most prominent at 27 DPI, as shown by the mean (± SE) trends of all DB829 and DB868 treatment groups (Figure 4). The average haematocrit (HCT) of the monkeys in the DB829 study (n = 6) was 35.5% (± 1.8) which was a 27.3% drop from the pre-infection mean value of 48.8% (±2.6). The monkeys in the oral DB868 study (n = 10) experienced a comparable 30.7% drop in mean haematocrit, from a pre-infection value of 42.4 (±1.5) to 29.4 (± 1.2) at 27 DPI. The mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) also declined significantly ($p < 0.05$), consistent with previous findings in monkeys infected with this strain (Thuita *et al*., 2012). Upon treatment with IM DB829 or oral DB868, the RBC counts recovered to baseline levels within approximately 1-2 months (Figure 4). In the monkeys that experienced trypanosome recrudescence in the blood (monkey 687 (DB868, 3mg/kg), monkeys 688, 695 and 697 (DB868, 10 mg/kg) and monkey 689 (DB868, 20 mg/kg) (Figure 2), a second phase of declining RBC parameters was observed concurrently with the reappearance of trypanosomes in blood (data not shown). No such decline in RBC associated parameters

was observed for monkeys 573 and 696 in which trypanosomes reappeared only in the CSF.

Infection related thrombocytopenia was observed between 7-27 DPI, followed by recovery to baseline (pre-infection) levels within one week after therapeutic intervention (Table 2). Similarly, a significant leucopenia was observed at 7 DPI (Table 2). This leucopenia was largely determined by significant declines in lymphocyte numbers (Figure 5). However, unlike RBC and platelet counts which remained low as long as trypanosomes were still in the blood, white cell densities increased in some individuals and remained low in others (Table 2, Figure 5). Upon treatment with DB829/DB868, blood white cell counts remained somewhat elevated in those monkeys that eventually relapsed after variable periods of post treatment monitoring (Figure 5). The strongest peaks of white blood cells were observed approximately 1-4 days post end of treatment with melarsoprol of relapsed individuals (Figure 5). These relapse-melarsoprol treatment associated peaks of blood white cells were mainly the result of increases in lymphocytes and granulocytes (Figure 5) and were observed only in those individual with trypanosomes in the blood but not those with only CSF trypanosomes.

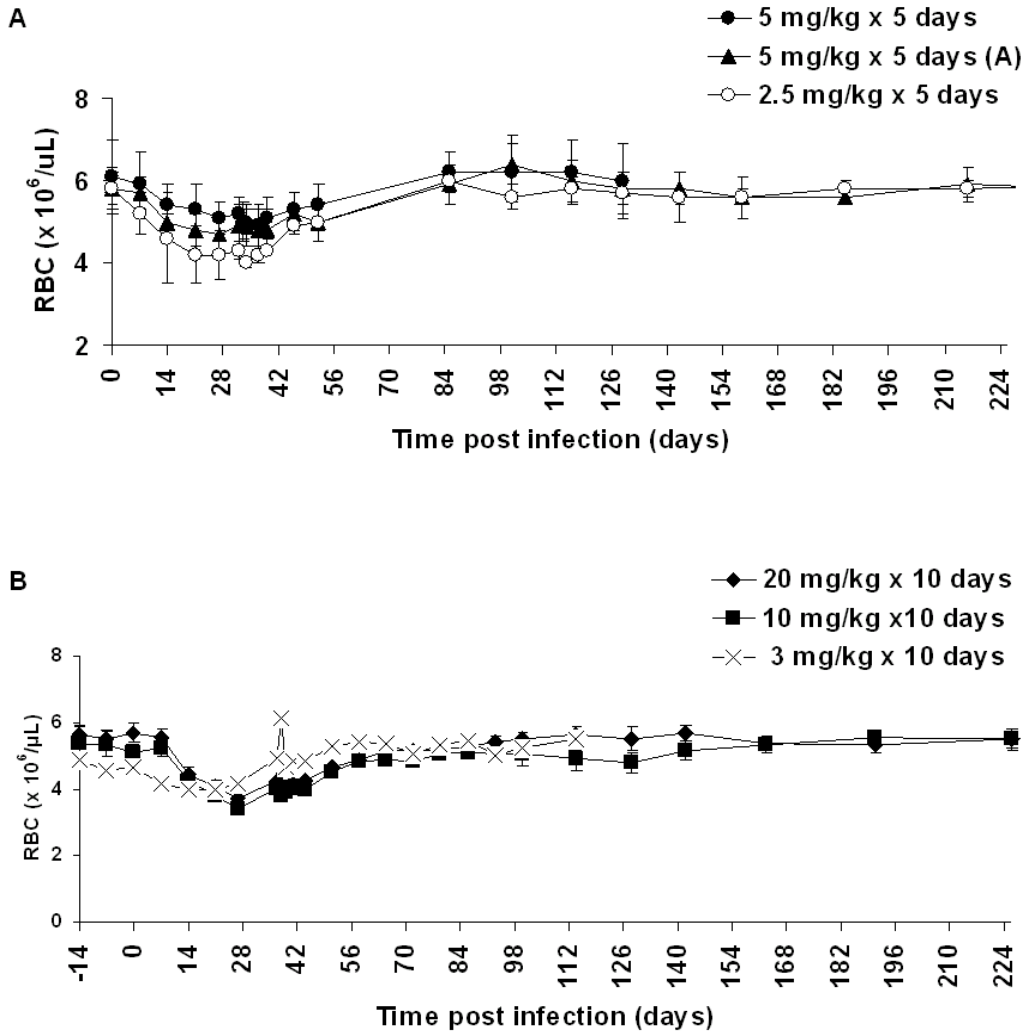

Figure 4: Changes in density of red blood cells in blood of monkeys that were infected with *T.b. rhodesiense* and later treated with IM DB829 (A) or oral prodrug DB868 (B). DB829 was administered from 28 days post infection for 5 consecutive days or on alternate days (A). DB868 was dosed orally for 10 consecutive days, starting 28 days post infection.

Table 2: Changes in blood platelet and white cell counts following infection of monkeys with *T.b. rhodesiense* and subsequent treatment with oral DB868 and intramuscular DB829.

Drug	Dose (mg/kg x d)	DPI (DPT)	0	7	27	38 (1)	44 (7)	65 (28)	100 (63)	137 (100)	224 (180)
Oral DB868	20 x 10 [a]	PLT	323 ± 42	112 ± 15 (S)	131 ± 32 (S)	410 ±14	362±57	334±52	318± 31	285± 51	243±50
		WBC	8.1± 1.5	3.5 ± 0.8 (S)	4.3 ± 0.7 (S)	7.4 ± 0.8	6.5± 0.6	5.9 ± 0.3 (S)	6.9 ± 0.6	8.1 ± 0.6	9.7± 2.9
	10 x 10[a]	PLT	273 ± 76	75 ± 13	54 ± 4	292 ± 41	331 ± 90	307 ± 82	217 ± 32	451 ± 65	337 ± 19
		WBC	5.7 ± 0.3	2.7 ± 0.2 (S)	4.3 ± 0.6	8.9 ± 0.3	7.1 ± 0.8	6.9 ± 0.7	7.9 ± 1.7	6.2 ± 0.5	6.4 ± 0.6
	3 x 10[a]	PLT	355 ± 215	259 ± 186	126 ±33	472 ±248	NA				
		WBC	5.7 ± 0.8	4.7 ± 0.6	6.4 ± 0.5	8.2 ± 0.6	NA				
	5 x 5[a]	PLT	424 ± 60	112 ± 11 (S)	156 ± 42 (S)	464 ± 32	515 ± 39	419 ± 4.5	352 ± 44	367 ± 60	NA
IM DB829		WBC	6.0 ± 0.4	3.4 ± 0.5	4.6 ± 0.2	7.8 ± 0.3	6.6 ± 0.5	7.3 ± 1.0	5.3 ± 1.1	4.5 ± 0.1	NA
	5 x 5[b]	PLT	312 ± 31	96 ± 12 (S)	200 ± 2 (S)	328 ± 7.5	395 ± 26	378 ± 71	246 ± 26	327±25	250 ± 53
		WBC	4.8 ± 0.5	3.3 ± 1.1	4.7 ± 0.6	4.8 ± 0.6	5.6 ± 0.5	6.5 ± 0.7	5.8 ± 0.8	6.3 ± 0.3	6.0 ± 0.3
	2.5 x 5[a]	PLT	285 ± 23	54 ± 20 (S)	115 ± 32 (S)	265 ± 17	332 ± 27	292 ± 5	242 ± 64	327 ± 27	389 ± 13
		WBC	5.8 ± 2.1	3.9 ± 1.4 (S)	5.4 ± 1.3	6.8 ± 1.5	8.9 ± 3.4	8.1 ± 2.1	6.3 ± 1.0	6.0 ± 1.6	6.7 ± 1.9

IM, intramuscular; d, day; a, consecutive day dosing; b, alternate day dosing; DPI, days post infection; DPT, days post last treatment; WBC, white blood cell counts; PLT, Platelets; NA; not applicable; S, significant at $p < 0.05$, repeated measures ANOVA and post hoc tests;

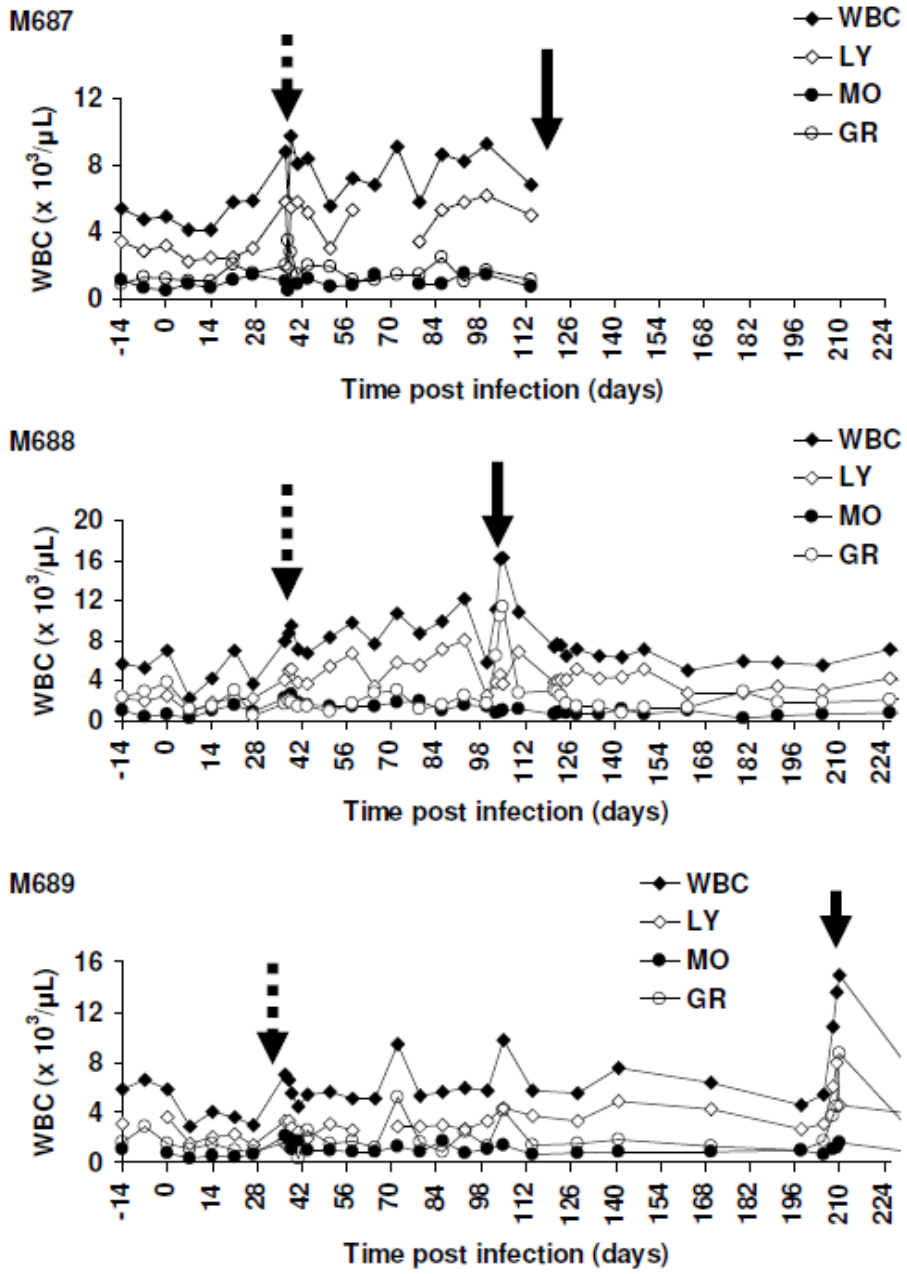

Figure 5: White blood cell changes in three monkeys that were treated with oral DB868. DB868 was administered orally at 3 mg/kg x 10 days (M687); 10 mg/kg x 10 days (M688); and 20 mg/kg x 10 days (M689). When relapses were confirmed, the monkeys were retreated with melarsoprol at 3.6 mg/kg x 4 days intravenously; WBC, white blood cells; Ly, lymphocytes; Mo, monocytes; GR, granulocytes. Dashed arrows: last dose of DB868; bold arrows, last dose of melarsoprol.

Pharmacokinetics

Following intramuscular injection of the active drug DB829 at either 5 or 2.5 mg/kg, similar T_{max} values of 0.04 days (1 h) were obtained for all six monkeys, showing that the drug was rapidly absorbed into the blood. A geometric C_{max} value of 13400 (12000-15000) nmol/L was obtained for the 5 mg/kg x 5 days consecutive day dosing group while 5000 (4200-5900) nmol/L was obtained for the 2.5 mg/kg DB829 dose group. (2.7 fold). In the alternative day dosing regimen at 5 mg/kg, C_{max} and T_{max} were comparable to the consecutive day dosing (Table 2). The AUC for the 5mg/kg group was 2.1-fold greater than that for the 2.5 mg/kg dose group. The terminal half-life for the consecutive day dosing regimens was an average of 38 days (32-44). The AUC and the terminal half-life for the alternative day dosing regimen were 5-fold and 3-fold greater than corresponding values for the consecutive day-dosing regimen at the same 5 mg/kg dose.

For monkeys dosed with oral DB868, the prodrug was detected within 1 h post dosing in all monkeys at geometric mean values of 812 nmol/L (20 mg/kg group), 746 nmol/L (10 mg/kg group) and 124.9 nmol/L (3 mg/kg group). The plasma values of the prodrug declined rapidly and were not detected after 8 h in any monkey. Thus, although the geometric mean values obtained at 1h were the highest plasma values for all the groups, accurate PK parameters could not be derived due to limited data points. On the other hand, the plasma T_{max} for the active metabolite DB829 was an average of 0.33 days (8 h) for the three treatment groups, with a range of 0.04-2 days (1 – 44 h) (Table 3). The C_{max} values were 526 (460-630) nM for the 20 mg/kg dose group, 269 (180-410) nmol/L for the 10 mg/kg dose group and 85 nmol/L for the 3 mg/kg dose group.

CSF active drug (DB829) concentrations were determined only for the two groups of monkeys that were treated with DB829 at 5 mg/kg x 5 days IM. In the consecutive day dosing group, the geometric mean DB829 concentration was 13.6 nmol/L at the 1 h post LDD time point; the levels declined to about 5.7 nmol/L but were detected until 21-28 days post LDD. In the alternate day dosing group, geometric mean DB829 concentration was 7.3 nmol/L at 1 h post LDD time point. The drug was detected at the same level until 2 days post LDD before falling below the limit of quantification of the assay of 5 nmol/L.

Table 3: Pharmacokinetics of DB829 in vervet monkeys with late stage HAT after the 5th intramuscular dose

Outcome	Units	Group 1		Group 2		Group 3	
		569	659	668	676	546	693
T_{max}	day	0.04	0.04	0.04	0.04	0.04	0.04
C_{max}	µmol/L	12	15	16	8.9	4.2	5.9
AUC_{last} †	µmol/L•day	12	13	97	35	7.7	4.5
$AUC_{0-\infty}$	µmol/L•day	13	15	120	40	8.3	5.3
Cl/F	L/day/kg	0.85	0.76	0.09	0.28	0.67	1.0
$t_{½}$	day	34	44	130	180	33	42

Group 1 = 5 mg/kg x 5 consecutive days; Group 2 = 5 mg/kg x 5 alternating days; Group 3 = 2.5 mg/kg x 5 consecutive days; T_{max}, time to reach C_{max}; C_{max}, maximum concentration; AUC_{last}, AUC from time 0 to the last measurable concentration; $AUC_{(0-\infty)}$, AUC from 0 to infinite time; Cl/F, apparent clearance; $t_{½}$, terminal half-life; NC, not calculable

Table 4: Pharmacokinetics of DB829 in vervet monkeys with late stage HAT after the 10th oral dose of DB868

	Monkey	T_{max}	C_{max}	AUC_{0-75}	$t_{½}$
		day	µmol/L	µmol/L•day	day
20mg/kg x 10 days PO	573	1	0.46	8.1	48
	679	0.33	0.63	14	NC
	689	0.17	0.50	4.9	180
	696	0.17	0.53	6.2	NC
10mg/kgx 10 days PO	688	0.04	0.41	2.5	NC
	690	2.0	0.34	5.5	120
	695		0.18	12	NC
	697	0.33	0.21	NC	NC
3mg/kg x10days PO	670	0.33	0.06	NC	NC
	687	0.33	0.11	2.1	NC

PO, per os; T_{max}, time to reach C_{max}; C_{max}, maximum concentration; AUC_{0-75} AUC from 0 to 75 days; $t_{½}$, terminal half-life; NC, not calculable; PO, per os

DISCUSSION

A variety of novel diamidines and their prodrugs have been synthesised (Das and Boykin, 1977; Ismail *et al*, 2003; Tidwell and Boykin, 2003) and subsequently shown to be highly active against African trypanosomes in vitro and in mouse models (Wenzler *et al* 2009; Ward *et al*., 2011). More recently, it was shown that one of the prodrugs, DB868, was significantly better tolerated in rats than the clinical candidate prodrug pafuramidine (Wolf *et al*., 2012). DB868 was also well tolerated in un-infected vervet monkeys and was highly active against first stage HAT in the vervet monkey HAT model, highlighting it as a potential new clinical candidate for first stage HAT (Thuita *et al*., PLoS NTDs, in press). In the current study, we compared the pharmacology (efficacy, pharmacokinetics) of orally administered DB868 to that of its intramuscularly administered parent drug (DB829) in the CNS stage vervet monkey model of HAT, in an effort to identify a suitable potential clinical candidate for the CNS stage human disease.

The diamidine DB829, when administered IM at 5 or 2.5 mg/kg for five days consecutively or at 5 mg/kg x 5 days on alternate days, cured all monkeys with a CNS stage infection. These results were an indication of its superior efficacy compared to other previously evaluated diamidine prodrugs such as pafuramidine which cured 0/3 (Mdachi *et al*., 2009) and DB844 which had a modest efficacy of 3/7 (43%) against similar CNS-stage infections in the monkey model (Thuita *et al*., 2012). The efficacy of IM DB829 was also in contrast to that of IM pentamidine which was shown to have good activity (2/3) in early stage disease (Thuita *et al*., PLoS NTDs, in press) but was not effective (0/3) when initiation of treatment was delayed to 14 days post infection with *T.b. rhodesiense* KETRI 2537 (unpublished data TRC KARI). In other studies, Schmidt and colleagues evaluated the efficacy of diminazene aceturate and several experimental diamidines against CNS-stage *T.b. rhodesiense* infections and reported unsatisfactory results (Schmidt and Sayer, 1982). The results we have reported therefore indicate that, unlike other diamidines and diamidine prodrugs previously evaluated in this monkey model, DB829 could potentially be developed as a therapy for CNS stage HAT.

Oral DB868 had an excellent (4/4) end of treatment provisional cure rate for both 10 and 20 mg/kg dose groups but not for the 3 mg/kg dose group (0/2). However, for the

10 and 20 mg/kg dose groups, the final efficacy result after more than 300 days of post-dosing monitoring was only 1/4 (25%) cure rate. In contrast, both DB868 and DB844, when administered at 100 mg/kg x 5 days per os, cured 4/4 GVR 35 CNS stage infections in mice (Wenzler *et al.*, 2009). A closer scrutiny of the treatment regimens used in the monkey and mouse studies suggests that the superior efficacies in the mouse model were likely related to the significantly higher daily drug doses employed (100 mg/kg [mouse] vs. 20 mg/kg [monkey]). Species differences (mouse vs. monkey) and the requisite scaling down of drug doses between species as well as results of toxicity studies of 10-day oral DB868 regimens in un-infected vervet monkeys (Thuita *et al*., PLoS NTDs, in press) were considered when selecting the maximum dose of 20 mg/kg x 10 days for the monkey efficacy studies. However, on the evidence of our current results and comparable partial cures obtained after prodrug DB844 was used to treat CNS stage infections in monkeys using 10 or 14-day dose regimens (Thuita *et al*., 2012), there may be need to investigate alternative treatment regimens comprising of higher daily drug doses which are administered for shorter durations to maximise on efficacy and minimise the risks of dose-limiting toxicity.

Pharmacokinetic evaluation of plasma samples provided evidence of the superiority of IM as compared to oral administration of these compounds. The geometric mean plasma C_{max} resulting from IM administration of DB829 at 5 mg/kg was 26 times (13400/512) greater than the active metabolite (DB829) concentration resulting from administration of oral DB868 at 20 mg/kg. Even when DB829 was dosed at 2.5 mg/kg IM, resultant plasma DB829 C_{max} values were approximately 10 times (5000/512) greater than values of the same compound (DB829) resulting from oral dosing of prodrug DB868 at 20 mg/kg. In terms of systemic availability of active drugs therefore, the comparatively poorer performance of orally administered prodrugs in comparison with IM administered active drugs is best explained by a report that 50–70% of an oral dose of pafuramidine was absorbed (losses: 30-50%) and effectively converted to the active drug (DB75) in both rats and monkeys but subject to first-pass metabolism and hepatic retention, limiting its systemic bioavailability to 10 to 20% (Midgley *et al*., 2007).

The fact that high efficacies were obtained in IM treated monkeys indicates that sufficient quantities of the active drug (DB829) crossed the blood brain barrier (BBB) to eliminate trypanosomes from the central nervous system (CNS). Evaluation of CSF samples from monkeys that were treated at 5 mg/kg x 5 days confirmed that DB829 was indeed detected, with a geometric mean of 13.6 nmol/L (consecutive day-dosing group) and 7.3 nmol/L (alternate-day dosing group) at 1 h post LDD. These CSF DB829 concentrations were not particularly high, especially in comparison with plasma levels of the same drug (Table 3). However, DB829 was present in CSF at levels greater than 5 nmol/L for at least 2 days post LDD (alternate day dosing group) and 21-28 days post LDD (consecutive day dosing group). It is proposed therefore that the presence of DB829 in CSF for relatively long periods led to successful elimination of trypanosomes from the CSF/CNS compartment, possibly due to the ability of trypanosomes to accumulate potentially toxic active drug levels from surrounding medium (Mathis *et al*., 2006). CSF DB829 levels were not determined for monkeys that were treated with oral DB868; the fact that poor overall efficacies were observed suggested that therapeutic concentrations of the active drug (DB829) were not attained in the CSF and CNS parenchyma of a majority of the monkeys. Studies on the related prodrug, pafuramidine reinforced the superiority of parenteral as compared to oral dosing of diamidines: active drug (DB75) concentrations were higher (61 nmol/mg of tissue) in the CNS of mice after IV administration of pafuramidine as compared to orally dosed mice (13nmol/mg of tissue) (Sturk *et al*., 2004). Thus, although efflux transporters present in the BBB (Krajcsi *et al.*2012) make it difficult for significant levels of active diamidines/other drugs to accumulate in the CSF/CNS, our data show that parenteral dosing improved active drug levels in CSF possibly through mechanisms such as concentration dependent diffusion (Nau *et al*., 2010) and/or enhanced activity of organic cation transporters (Ming *et al*., 2009).

Monkeys that relapsed at various times after oral DB868 treatment manifested clinical and clinico-pathological parameters that were distinctly different from primary infections in the same individuals. Firstly, the clinical disease in relapsed individuals was characterised by more signs of late CNS stage disease than primary infections in the same

monkeys, evidence of the important role of CNS as source of relapse trypanosomes (Thuita et al 2012). Secondly, parasitaemia in the relapsed monkeys hardly reached the peak levels observed in primary parasitaemia and was actually more similar in pattern to that seen in *T. b. gambiense* infections in monkeys (Dr David Mwangangi, personal communication) and in sleeping sickness in humans which is largely (more than 95%) caused by *T.b. gambienese* (Brun *et al*., 2011; WHO, 2012; Matovu *et al.,* 2012). Parasitaemia loads in *Trypanososma brucei spp* are determined by density dependent parasite differentiation to non-replicating short stumpy forms and parasite killing by the hosts immune system (Tyler *et al*., 2001). The apparently poor growth characteristics of relapse as opposed to primary infections of the same trypanosomes were therefore likely indicative of a heightened recognition and faster removal of successive variant antigenic types (VATs) by the immune system in the infected monkeys. The trypanosomes regained their robust growth characteristics and high parasitaemia loads when they were passaged in naive rodents (unpublished observations), indicating that there was no permanent change in trypanosome phenotype. Due to their intermittent nature, relapse parasitaemia were more difficult to detect by microscopy (van Meirvenne *et al*., 1999), sometimes only being detected when clinical disease was well developed. Importantly, confirmed relapse infections were successfully retreated with intravenously administered melarsoprol at 3.6 mg/kg x 4 days, highlighting the importance of this old drug in the fight against HAT, despite the well documented toxicity issues associated with its use (Burri, 2010; 2012)

A major constraint of preclinical drug studies in animal models is the requirement for long post treatment monitoring periodes (more than 300 days in CNS stage HAT monkey model) before cure can be declared. The timelines are even more protracted for clinical trials of potential new HAT treatments, with at least 18-24 months needed to confirm cure (WHO, 2004). However, our time to relapse data for monkeys treated with DB868 at 20 mg/kg, a median 133 (range, 77-161) days post last drug dose (LDD), indicates that the protracted timelines are not without justification. In an effort to improve the turnover times for compounds in development, primary efficacy endpoint assessments at 100 days post LDD have been proposed (Sonja Bernhard, personal communication). In our study, however, a primary efficacy endpoint set at 100 days post LDD would have

correctly predicted the final efficacy result for oral DB868 at 10 mg/kg group but not for the 20 mg/kg group i.e. 2/3 relapses in this group were diagnosed as relapses after the 100 days post LDD time point. Indeed, a review of larger data sets of monkeys treated with different experimental diamidines in our laboratory revealed that a primary endpoint at 100 days post LDD would have facilitated detection of 80% of all animals that eventually relapsed (unpublished data of TRC-KARI), highlighting a potential pitfall of reliance on primary efficacy data for decision making. Clearly, the utility of primary endpoint efficacy assessments needs to be improved, possibly by combining parasitaemia determinations with other surrogate biomarkers of cure assessment including CSF white cell counts (Priotto *et al*., 2012), serum/plasma trypanosome antigen levels as determined using TrypTectCIAAT (Karanja *et al*., 2010). However, these surrogate tests still need to be adequately validated to be reliably used as biomarkers of cure/relapse in drug trials.

Haematology changes attributable to trypanosome infection and subsequent treatment with diamidines were generally consistent with previous observations in this model (Mdachi *et al*., 2009; Thuita *et al*., 2012; Maina Ngotho *et al.,* 2011). In the current study, however, we focussed more on total and differential blood white cell counts and observed that this parameter remained elevated in monkeys which eventually relapsed, suggesting this parameter could be a potential surrogate marker for cure assessment. In addition, blood white cell counts, especially lymphocytes and granulocytes, were markedly elevated post treatment with melarsoprol. The leucocytosis was likely a sequel to rapid destruction of trypanosomes by melarsoprol, release of high amounts of trypanosome antigens (antigenaemia) and their stimulatory effects on the host's immune system. Importantly, the elevated leucocyte numbers normalised within approximately four days post LDD (Figure 5) and remained at baseline levels for the duration of post treatment monitoring; all other parameters remained normal indicating that like IM DB829, melarsoprol was curative for monkeys with CNS stage HAT.

Overall therefore, this study has identified IM DB829 at 2.5 mg/kg administered for 5 consecutive days, but not oral DB868, as a promising late lead compound that could enter the development pipeline as a new therapeutic agent for CNS stage HAT. Despite

the fact that prodrugs cross biological membranes like gastrointestinal mucosa better than active diamidines (Ansede *et al*., 2004), we have demonstrated conclusively that parenteral administration of diamidines is superior to oral administration of diamidine prodrugs in terms of eventual systemic availability of active drugs, thus leading to better efficacies against the tissue invasive HAT causing parasites.

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Chapter 8: General Discussion

The Case for a New HAT Drug

There is considerable concurrence among stakeholders on the need for new sleeping sickness medicines. Several authors, including those from the WHO, have published repeatedly on the need for new tools (especially drugs and diagnostics) in order to better meet the healthcare needs of sleeping sickness patients as well as other NTDs (Werbovetz *et al*., 2006; Simmaro *et al*., 2008; 2011; Brun *et al*., 2010, 2011; Chatelain and Ioset; 2011). The drive for new drugs is largely based on the well documented shortcomings of currently available treatments (Burri, 2010) and the need to increase the treatment options available for this disease to cater for emergent cases of parasite resistance/treatment failure. The studies reported in this PhD thesis show that two new chemical entities (NCEs), an oral prodrug DB868 and its intramuscularly administered parent drug CPD-0802, could be recommended to enter regulatory development for first and second stage HAT respectively.

Currently, compounds undergoing regulatory development for HAT include fexinidazole (phase II/III) and the benzoxaborole SCYX-7158 (phase I). These compounds performed well during preclinical and early phases of clinical development (Nare *et al*., 2010; Toreele *et al*., 2010; Kaiser *et al*., 2011; Jacobs *et al*., 2011), thus generating considerable optimism on their chances of successfully completing the drug development process. Indeed, it is now believed that a new drug will become available within the next 3-5 years (Maser *et al*., 2012). However, the drug development process suffers from a considerably high attrition ratio, with only 1:50 000 NCEs advancing to clinical trials from the drug discovery phase (Grewal *et al*., 2008). Even after potential new therapies have entered clinical trial phase, the attrition ratio remains high. In a study by Prentis and Walker (1986), it was reported that out of 197 NCEs developed by seven UK pharmaceutical companies up to clinical testing phase, only 35 were eventually marketed giving an attrition ratio of 5.6:1. Comparable attrition ratios of 10:1 (Wardell and Scheck, 1982) and 13.5:1 (Mattinson *et al*., 1984) were reported for US and Swiss

companies respectively. Although the period since year 2000 has been very good for HAT drug research and development (R&D), the considerably high risk of attrition should remind us that with only two compounds currently, the HAT drug development pipeline is not yet robust enough.

In spite of the scientific evidence and my advocacy for advancing both diamidines to regulatory development, it must be mentioned that drug development is a very costly undertaking. Actual estimates of developing a new drug up to market authorisation range from USD 500-2000 million (Dimasi *et al*., 2003; Adams and Brantner, 2006) although this cost is likely to be lower for drugs developed by the not for profit public private partnerships (PPPs). In addition, it takes an estimated 10-12 years to develop a new drug (Grewal *et al*., 2008). On the basis of cost then, it is unlikely that both diamidines could actually end up in regulatory development- rather a best case scenario would see only one of the two drugs funded for development. If it came to a choice between the two, conventional thinking would favour the parent drug DB829 on the basis of its activity against second stage disease. This is shown by target product profiles (TPPs) in use by the consortium for Parasitic Drugs Development (CPDD) and Drugs for Neglected Diseases initiative (DNDi) (sample TPP: appendix) which usually include activity against second stage disease top of the list of desirable attributes of potential new therapies (http://www.dndi.org/diseases/hat/target-product-profile.html, Susan Jones, personal communication). However, the orally active drug DB868 would also have its supporters, including myself. I consider arguments for oral HAT medicines, presented in this thesis and elsewhere, quite compelling. A key plank in these arguments is that an oral medicine would make it possible to reduce hospitalization times and, in a best case scenario, completely render it unnecessary for a proportion of the patients. Such an outcome would have the ripple effect of reducing healthcare costs at the national level, thus freeing up financial resources to take care of other pressing healthcare concerns such as malaria. It would also free up more time for patients' relatives/attendants to engage in economic activities that strengthen the household economy, as compared to spending a lot of time taking care of hospitalized patients. In view of high poverty levels in disease

endemic countries, an efficacious oral drug would, in my view, be favourable even if it was only active against first stage HAT.

If a potential advantage of an efficacious oral take home medicine would be to reduce the proportion of patients requiring hospitalization, the question of which patient categories (first or second stage) could benefit most needs to be considered. An oral take home medicine, if available, is likely to be prescribed only for those patients who are not moribund or in any way so incapacitated as to make hospitalization mandatory. In general, first stage patients fit the description of those who are likely to benefit from such medicines better than second stage patients. It must also be mentioned that barriers to early diagnosis of HAT are progressively being removed. One of these barriers, war and conflict, contributes to the spread of HAT through displacement of large populations of people and their livestock some of which are infected carriers (Kuzoe, 1993; Ford, 2007). Insecurity also makes certain regions/villages in HAT endemic countries inaccessible to health personnel thus hindering efforts to carry out proper surveillance; in areas where active surveillance has been carried out effectively HAT cases are increasingly diagnosed while still in first stage (Chappuis *et al*., 2010). It is encouraging to note that major long running conflicts in Angola and South Sudan have been resolved recently and the security situation has also improved in the DRC. Another hindrance to early diagnosis of HAT has been lack or poor knowledge of the disease symptoms among the general population. This drawback has been innovatively addressed in Kenya through education of schoolchildren as a way to disseminate vital disease information to the community (Julia Karuga, personal communication). Primary healthcare workers have also been targeted for education in a campaign to improve early detection of HAT (Dr Grace Murilla, personal communication). While the impact of these initiatives has not been independently evaluated, it is believed that supplying the right information to those who need it should help to improve early case detection which in turn would increase the proportions of patients likely to benefit from an oral drug for 1st stage HAT.

Based on recent successes in reducing the incidence and prevalence of HAT, some authors currently believe that elimination of HAT as a disease of public health significance is an achievable goal (Nimo, 2010; Aksoy, 2011; Simmaro *et al*., 2011). In

order to achieve this noble goal, however, the stakeholders must find a way to keep the HAT control agenda alive despite the dwindling case numbers. We must find a way to avoid falling into a similar pitfall as happened in the 1960s when success was followed by a period of uncertainty and in some cases neglect of vital disease surveillance activities, leading to resurgence in the disease in the 1970s to 1990s (Simmaro *et al*., 2008; Nimo, 2010). The WHO needs to continue the excellent coordinating and motivating role it has had on National Disease Control Programmes (NDCPs) as well as being the link between drug manufacturers and HAT endemic countries. In addition, regional groupings such as the Eastern Africa Network for Trypanosomiasis (EANETT) and the HAT Platform need to continue to meet to facilitate sharing of vital disease control information between members. Importantly, however, the development of new drugs must continue to be pursued despite the increased difficulties of carrying out clinical trials (Burri, 2012) and the challenge of finding donors for HAT R&D in the face of the reduced case numbers.

Perhaps the most important reason why development of new drugs needs to remain a priority is the whole question of parasite resistance to existing agents. The case of resistance to melarsoprol is well documented (Maser *et al*., 1999; Brun *et al*., 2001; De Koning, 2001; Maina *et al*., 2006; Barret *et al*., 2011). In addition, the first anecdotal reports of treatment failures with eflornithine monotherapy are already emerging from some HAT foci (Barret *et al*., 2011). Resistance to melarsoprol and eflornithine (this would likely also affect NECT), would make it possible to get second stage HAT patients for whom there would be no available treatment - a scenario that must be avoided at all costs. However, the good news is that important developments have occurred in the chemotherapy of HAT in the recent past. These include the development and inclusion of the nifurtimox eflornithine combination therapy (NECT) in the essential medicines list of the WHO for the management of *T. b. gambiense* HAT (Priotto *et al.*, 2009; WHO, 2012). In addition, the ten-day melarsoprol treatment schedule that was already in use for *T. b. gambiense* HAT (Burri *et al*., 2000) has been evaluated against *T. b. rhodesiense* HAT in Tanzania and Uganda (Kuepfer *et al*., 2012). The results of these clinical trials indicated that the 10-day melarsoprol schedule had an efficacy of 96% at the 12 month

post treatment follow-up time point and did not expose patients to a greater risk of encephalopathic syndromes compared to the standard melarsoprol treatment schedules (Kuepfer *et al*., 2012). A number of molecules including the diamidines reported in this thesis and proposed new formulations of old drugs (Kennedy, 2012) have shown promise in preclinical development. The development of better treatment schedules of current drugs as well as new safe and efficacious treatments must remain a priority in order to "dim the lights (Nimo, 2010)" on HAT.

Understanding the Determinants of Diamidine Toxicity

DB289 (pafuramidine) has so far been the only new generation diamidine developed by CPDD to enter clinical testing. The clinical trials were conducted in the DRC, Angola and South Sudan. DB289 performed well in phase I/II clinical trials (http://clinicaltrials.gov/ct2/show/NCT00803933), and became the first oral drug to enter the pivotal phase III clinical trial where its efficacy was insignificantly different from that of intramuscular pentamidine (Paine *et al*., 2010; Burri, 2010, Maser *et al*., 2012). However, while the phase III clinical trial for HAT was still in progress, an extra phase I clinical trial was initiated in South Africa (SA) at the request of the Federal Drug Agency (FDA) of the USA in order to generate additional safety data to support registration of the drug for *Pneumocystis jiroveci* pneumonia. In this new study, DB289 was dosed at 100 mg/kg bid for an extended 14 days as compared to 5 (phase I/II) and 10 days (phase III) clinical trials for HAT (http://clinicaltrials.gov/ct2/results?intr=%22DB289%22; http://clinicaltrials.gov/ct2/show/NCT00619346?intr=%22DB289%22&rank=5). Liver and more importantly delayed kidney toxicity were observed in this new phase I study, necessitating termination of the DB289 development programme (Paine *et al*., 2010). The phase III clinical trials data was then unblinded at which point two out of three nephrotoxicity cases were identified as possibly related to DB289; no such cases were reported for pentamidine (Dr Allison Harril, personal communication). Significantly, though, nephrotoxicity was also not reported for phase I/II DB289 clinical trials for HAT. Naturally, the determinants of DB289 (and by extension diamidine) toxicity needed to be elucidated in order to aid selection of next-in-class compounds that could be safely used in humans.

Insights from the Monkey HAT Model

Pharmacological investigations carried out in vervet monkeys on DB289, DB844, DB1058, DB868 and DB829 suggested that members of the class (diamidines) exhibit differential toxicity profiles. Based on cumulative doses administered to un-infected monkeys during the toxicity study and cumulative doses that were curative in the 1st stage HAT model, DB868 exhibited a maximum tolerated dose (MTD): minimum curative dose (MCD) ratio of 14:1 (300:21). On the other hand, pafuramidine was administered at a maximum dose of 10 mg/kg x 10 days (CD = 100 mg/kg) and was fully curative (3/3) against 1st stage HAT when administered at 10 mg/kg x 5 days (Mdachi *et al*., 2009) indicating an approximate MTD: MCD ratio of 2:1. DB844, another of the prodrugs, turned out to have an insufficient therapeutic index for the targeted 2nd stage disease indication (Thuita *et al*., 2012), but was not evaluated against 1st stage HAT which, our studies on other prodrugs showed, require significantly less total drug doses. Similar to DB844, another prodrug DB1058 was poorly tolerated in un-infected monkeys (data not shown) and was therefore not evaluated for efficacy in the monkey model. The parent drug CPD-0802 was tolerated at 5 mg/kg x 5 days (CD = 25 mg/kg) and was fully curative at 2.5 mg/kg x 5 days (CD = 25 mg/kg) giving an apparent therapeutic index of 2:1 for 2nd stage HAT. On the basis of these data, oral DB868 had the best therapeutic index when used against 1st stage HAT and would therefore be an interesting candidate for development. The five compounds (Figure 1) are structurally very similar; they differ only in the number and location of nitrogen atoms in their phenyl rings (appendix 1). The observed differences in their activity profiles highlight the value of structure activity relationship studies (SARs) as a strategy for filtering out the poor compounds and allowing potentially beneficial ones to progress to the next phase of the drug development process.

In addition to differences between compounds, compound dose and duration of dosing were identified as important determinants of toxicity (e.g. for DB844 in Thuita *et al*., 2012). These studies relied on standard techniques, including observation for overt toxicity, haematology, and assay biomarkers of liver (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) and kidney (blood urea nitrogen [BUN] and

creatinine [CREA]) toxicity. In addition, animals that needed to be euthanized were subjected to necropsy and histopathology. A wider array of newer biomarkers especially for kidney toxicity are available and have been used to study diamidine toxicity in rodents (discussed below) but these were not used for the monkey studies. Even with the standard techniques employed in the monkey studies, however, the relationship between dose and toxicity was clearly demonstrated for the different compounds described in this thesis. In my view, these findings may partly explain why more pronounced toxicity, including delayed kidney toxicity, was observed in human volunteers who received a higher cumulative dose of pafuramidine (100 mg BID x 14 days) as compared to those who received the lesser doses (100 mg BID for 10 days or less) during the clinical trial phase for HAT. During the phase I/II DB289 clinical trials, all the volunteers and/or patients were assessed to have tolerated the drug well, with only mild and self-limiting side effects whose frequency and severity were comparable in the pafuramidine-treated and placebo or comparison drug treatment (pentamidine) group (Immtech data cited in Nyunt *et al*., 2009). In the clinical setting, adverse drug reactions (ADRs) that exhibit clear dose dependence and can be reproduced in experimental animals are termed "Type A" ADRs (Thompson *et al*., 2012). These types of ADRs can theoretically be avoided by identifying and keeping below the "threshold" for toxicity which, on the evidence of toxicity and efficacy studies in monkeys, would be best achieved through relatively short dosing regimens of 5-7 days duration. A recent clinical trial (ISRCTN55042030) attempted to reduce dosing for pentamidine to three days compared to the standard 7 to 10 day period at 4 mg/kg (Burri 2010) based on previous pharmacokinetic studies of pentamidine in *T. b. gambiense* patients (Bronner *et al*., 1991).

At clinical chemistry level, diamidine toxicity in monkeys was characterised by changes in the standard biomarkers of both liver and kidney, similarly to the case in humans. This is not surprising, considering the phylogenetic closeness between human and non-human primates. Aberrations were demonstrated in either ALT or AST for the liver as well as blood urea nitrogen (BUN) or creatinine for the kidney. In my view, the challenge for drug developers is to decide whether transient 2-3 fold increases in clinical chemistry analytes (e.g. blood urea nitrogen) constitute a significant safety concern to

warrant discarding of a new chemical entity. Achieving the balance between essential safety concerns and the possibility that promising therapeutic agents will get discarded without adequate review of their potential is not easy. It must be said also that pentamidine, which continues to be of benefit to thousands of HAT, leishmaniasis and *Pneumocystis jiroveci* pneumonia does not only cause some degree of reversible liver damage but it also causes some nephrotoxicity (Bronner et al., 1991). In conclusion, the observations in monkey studies suggest that the new diamidines are not more toxic than pentamidine; dose and duration of dosing are the twin issues that must be carefully considered in regulatory development of a next-in- class diamidine.

Insights from the Mouse Diversity Panel (MDP) and Rats

Efforts to understand how diamidines can be safely used therapeutically have also involved studies in rats (Wolf et al., 2012) and the development of a new model, the mouse diversity panel (MDP) by Dr Allison Harril and colleagues at the Hamner Institute for drug safety studies, University of North Carolina (UNC). In their studies, Kristina Wolf and colleagues compared the toxicity of DB289 and DB868 in female Sprague-Dawley rats (8-10weeks). The results signify differences in the toxicity of the two compounds. D289 was found to have caused a 12-fold increase in mean ALT concentrations in comparison with vehicle (carboxymethyl cellulose), while DB868 caused no such elevations of ALT. In their study, Wolf and colleagues found no changes in the standard biomarkers of kidney toxicity (BUN and CREA) that could be attributed to either DB289 or DB868. Novel biomarkers of kidney injury including the kidney injury molecule 1 (KIM-1) and full name TIMP I were found to be elevated in DB289 treated rats, suggesting that these were more sensitive markers of renotoxicity in this model.

A new rodent tool, the mouse diversity panel (MDP) was additionally evaluated for ability to predict renal toxicity (Dr Allison Harrill, personal communication). Dr Harrill and colleagues at the Hamner-University of North Carolina Institute for Drug Safety Sciences developed the MDP based on the hypothesis that genetic variability may render certain individuals/populations more susceptible to renal injury. This hypothesis

arose out of the fact that considerable DB289 renal toxicity was detected only after the extended phase I clinical trial in SA but not during clinical trials held in the DRC, Angola and South Sudan. In their studies, Harril and colleagues dosed 34 different strains of mice with DB289 and found that there were differences in susceptibility among the different mouse strains. They noticed that some strains of mice were indeed more susceptible to DB289 toxicity and could therefore be very useful in predicting clinically relevant toxicities in the evaluations of next-in-class molecules. Harril and colleagues also found that the KIM-1 and TIMP were more sensitive biomarker than standard renal clinical chemistry biomarkers-urea and creatinine. The addition of the MDP to an array of models available for preclinical evaluation of promising drug compounds makes it possible to detect agents that may cause toxicity only in certain susceptible patients. When toxicities are detected only in certain susceptible patients in the clinical setting (human), they are referred to as idiosyncratic drug reactions (IADRs, or Type B) (Thompson *et al*., 2012).

Improving the Efficiency of Drug Evaluations in the Monkey Model

Preclinical models are a critical component of R&D programmes. One key area of concern has been the need to monitor the monkeys for at least 300 days post last dosing in order to confirm cure which in turn slows down turn-atime for the compounds in the model. We hypothesised that loop mediated isothermal amplication (LAMP) of trypanosome DNA could be a useful test that would help in reduction of post treatment monitoring times. Preliminary studies on biological samples collected from the monkeys of the efficacy studies indicated that LAMP was indeed very sensitive in detecting residue DNA in biological fluids. However, clearance of the DNA post treatment appeared to be erratic in different individuals, in some cases DNA was detected through out the post treatment monitoring period. This result was essentially similar to a report that PCR detected DNA in patients throughput the period of post treatment monitoring (Matovu *et al*., 2012) suggesting that DNA based tests may be good for diagnosis of new infections but not so good for detection of cures or relapses post treatment.

In our studies we found that approximately 80% of monkeys that eventually relapsed had already been detected by the 3 months post treatment time point (appendix

2). This observation suggested that where several compounds were available to be tested, the result at 3 months could be used as a milestone to decide whether to initiate a new study in the monkey model or not which would serve to improve turnover time for late stage drugs. This approach could be applied until more sensitive biomarkers have been identified that could reliably be used to predict cure. Work done so far on an antigen based test, TrypTect CIATT showed that about 12 months were needed before antigens were totally cleared from serum and CSF of cured monkeys and humans (Karanja *et al*., 2010). The search for new biomarkers that can reduce post treatment follow-up has not yet yielded results that could be used in drug trials. In the meantime, our results on the 3 month milestone for efficacy evaluations in the model could help to improve efficiency in preclinical monkey model studies.

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Chapter 9: Acknowledgements

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CHAPTER 10: CURRICULUM VITAE

Name	Dr John Thuita Kibuthu
Address:	Trypanosomosis Research Centre, Kenya Agricultural Research Institute (TRC-KARI), P.O. Box 362, code 00902, Kikuyu, Kenya
E-mail:	thuitajk@yahoo.com; Johnthuita.kibuthu@unibas.ch
Telephone:	(020) 2700654 (office); 0721638122 (mobile)
Date of birth	7-8-1962
Nationality	Kenyan
Religion:	Christian
Marital status:	Married with 2 children

Educational Details

2009-2012	PhD studies at the Swiss Tropical and Public Health Institute and University of Basel, Switzerland.
1989-1992	Masters of Veterinary Public Health (M.V.P.H.), University of Nairobi (UON), Kenya
1985-1989	Bachelor of Veterinary Medicine (B.V.M.) University of Nairobi, Kenya
1981-1983	Kenya Advanced Certificate of Education (K.A.C.E.), Njoro High School. Obtained 3 Principals and 1 Subsidiary
1977-1980	Kenya Certificate of Education (K.C.E.), Njoro High School. Obtained a 1st Division of 15 points

Employment History

September 2009- Current:	Senior Research Officer (SRO) in Trypanosomiasis Research Centre, Kenya Agricultural Research Institute

(TRC-KARI). Main duties: pre-clinical chemotherapy studies of human anti-trypanosomal drugs.

April 2000- September 2009: Research Officer in TRC-KARI

1991 –April 2000: Veterinary officer in the Department of Veterinary Services, Kenya.

Grants/Research Projects

- Consortium for Parasitic Drugs Development (CPDD), responsible for in vivo studies of new compounds in the monkey model of HAT. Funding: CPDD
- Evaluation of Temozolomide for safety in the monkey HAT model. Funding: Private sponsor

Research Publications

- **Thuita JK**, Wang MZ, Kagira JM, Denton CL, Paine MF, et al. (2012) Pharmacology of DB844, an Orally Active aza Analogue of Pafuramidine, in a Monkey Model of Second Stage Human African Trypanosomiasis. PLoS Negl Trop Dis 6(7): e1734. doi:10.1371/journal.pntd.0001734
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Posters/ Conference Proceedings

- Tanja Wenzler, **John Thuita**, Mohamed A. Ismail, Donald A. Patrick, David W. Boykin, Richard R. Tidwell, Reto Brun. Novel diamidines for 2nd stage African sleeping sickness: new preclinical drug candidates. Emerging Paradigms in Anti-Infective Drug Designs" in London, UK: 17 – 18. September 2012.
- Tanja Wenzler, **John Thuita**, Donald A. Patrick, Richard R. Tidwell, David W. Boykin, Reto Brun (2012). Diamidines for 2nd stage African sleeping sickness:new preclinical drug candidates. Annual Swiss Trypanosomatid Meeting in Leysin, CH: 25th – 27th January 2012
- Tanja Wenzler, **John Thuita**, Mohamed A. Ismail, Donald A. Patrick, David W. Boykin, Richard R. Tidwell, Reto Brun. Novel diamidines for 2nd stage African sleeping sickness: new preclinical drug candidates. Biovalley Science day (MipTec) in Basel, CH: 20. September 2011
- Tanja Wenzler, **John Thuita** and Reto Brun. Diamidines for 2nd stage sleeping sickness. Presented during the Swiss Trypanosomatid Meeting in February 2011. Leysin, Switzerland
- **John Thuita** Grace Murilla and Reto Brun *et al*. Prospects for treatment of human African trypanosomiasis with novel diamidines: studies in the vervet monkey model of HAT. Presented during the 11th annual HAT Platform/EANETT scientific conference on 4-5th Oct 2010 at the Silver Springs Hotel, Nairobi, Kenya.

Affiliations

- Registered by the Kenya Veterinary Board
- Member of Kenya Veterinary association

Responsibilities

- Head, Chemotherapy Division of the TRC-KARI (2012)
- Chairman, KETRI Christian union, 2002 to date.

Academic and Professional Referees

- Prof. Reto Brun, Swiss Tropical and Public Health Institute and University of Basel, Socinstrasse 57, CH-4002 Basel, Switzerland
- Dr. Grace Murilla, (MSc, PhD, Centre Director), Trypanosomiasis Research Centre (TRC), Kenya Agricultural Research Institute (KARI), P.O. Box, P.O. Box 362, 00902, Kikuyu, Kenya.
- Dr. Nguhiu Mwangi (BVM, MSc, PhD), Kabete Campus, University of Nairobi, P.O box, 29053, Nairobi