Discovery of novel active diamidines as clinical candidates against *Trypanosoma evansi* infection

Inauguraldissertation

zur
Erlangung der Würde einer Doktorin der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von
Kirsten Gillingwater
aus Hornussen, AG

Basel, 2007

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel auf Antrag der

Herren Prof R. Brun, Prof P. Büscher, Prof C. Gutierrez und Prof J. Ed. Hall

Basel, den 22. Mai 2007

Prof HP. Hauri
Dekan

for my parents and marc

Do not go where the path may lead,
go instead where there is no path and leave a trail…

Ralph Waldo Emerson

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ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism and Excretion
Ag-ELISA	Antigen enzyme-linked immunosorbent assay
AIDS	Acquired immunodeficiency syndrome
AT-rich	Adenine - Thymine rich
AUC	Area under the curve
AUMC	Area under the first moment curve
BMEM	Baltz minimum essential medium
bp	Base pairs
CASY	Cell counter and analyser system
CATT	Card agglutination test for trypanosomiasis
C_{max}	Peak concentration
COST	European cooperation in scientific and technical research
DB	David Boykin
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTPs	Deoxynucleotide triphosphates
dTTP	Deoxythymidine triphosphate
EDTA	Ethylene diamine tetra acetic acid
GPI	Glycosyl-phosphatidyl-inositol
HAT	Human African Trypanosomiasis
HCT	Haematocrit centrifugation technique
HEPES	2-Hydroxyethylpiperazine-ethanesulfonic acid
HIV	Human immunodeficiency virus
HPLC	High pressure liquid chromatography
IC_{50}	50 % growth inhibitory concentration
IFAT	Indirect immunofluorescence antibody test
ILRI	International Livestock Research Institute, Kenya
i.p.	Intra-peritoneal
ITM	Institute of Tropical Medicine, Belgium
K	Kinetoplastic
K_{elim}	Rate of elimination constant
K.O.	Knock out
LDL	Low-density lipoproteins
mAECT	Mini-anion exchange centrifugation technique
MEM	Minimum essential medium

MRT	Mean residence time
MS	Mass spectrometry
NMRI	Naval Medical Research Institute
NTTAT	Non-tsetse transmitted animal trypanosomoses
OIE	Office International des Epizootes
OVI	Onderstepoort Veterinary Institute, South Africa
P2	Purine transporter 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPP	Public-Private Partnership
PSG	Phosphate buffered saline with glucose
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute
SIT	Sterile insect technique
SOP	Standard operating procedure
SPF	Specific pathogen free
sp.	Species
STIB	Swiss Tropical Institute, Basel
$t_{1/2}$	Half-life
T.b.	*Trypanosoma brucei*
T_{max}	Time at which peak concentration occurs
US$	United States of America, dollars
U.S.S.R	Union of Soviet Socialist Republics
UV	Ultra-violet
VSG	Variant surface glycoprotein

Summary

The animal pathogenic protozoan, *Trypanosoma evansi*, leads to a wasting disease in equines, cattle and camels, commonly known as Surra. It is extensively distributed geographically with a wide range of mammalian hosts and causes great economical loss, especially in areas of Africa, Asia and South America. Transmitted mechanically by biting flies (tabanids), the most effective form of control for this disease appears to be chemotherapy and treatment is currently based on four main drugs: suramin, diminazene aceturate, quinapyramine and cymelarsan. However, due to emerging resistance to these drugs, their toxicity and a sparse drug repertoire, their effective use is severely threatened, emphasising the urgent necessity in finding new alternative drugs.

Diamidines are dicationic molecules, which bind to the minor groove of DNA at AT-rich sites and then either inhibit one or more DNA dependent enzymes, or directly impede the transcription process. It has been known since the 1940s that these molecules contain anti-protozoal properties. The diamidine drug, pentamidine, is currently used to treat *Pneumocystis carinii* pneumonia, leishmaniasis and the early stage of African trypanosomiasis. Yet pentamidine is impeded by its toxicity, its inability to be absorbed orally and a limited understanding of its mode of action. New diamidine compounds were recently synthesised, revealing excellent *in vitro* and *in vivo* activity against human African trypanosomiasis.

The overall aim of this project was to explore the possibility of finding new diamidines with considerable activity against *Trypanosoma evansi* and to identify any novel diamidine compounds, which possess the potential to act as clinical candidates against this infection. Investigations carried out in this thesis using *Trypanosoma evansi* demonstrated outstanding *in vitro* activities for 181 diamidines, with IC_{50} values below 30 ng/ml. These compounds were further examined within an *in vivo* mouse model to determine the lowest curative dose and to ascertain preliminary toxicity values of the most active diamidines. A total of 49 compounds were examined for their *in vivo* efficacy, of which six were capable of curing *Trypanosoma evansi* infected mice at 0.5 mg/kg doses, given over four consecutive days. Additionally, a series of *Trypanosoma evansi* strains, isolated from various geographical locations, were also tested, to establish the effective sensitivity of such chemicals to different strains.

Based on these *in vitro* and *in vivo* findings, three novel diamidines (DB 75, DB 867 and DB 1192) were selected and tested for preliminary acute toxicity within a goat model. Several toxicity trials were performed, whereby a single, 4mg/kg dose of each compound was

given to goats and a multiple dose was given as four 1 mg/kg drug applications over six hours. All drug injections were given to the goats via an intramuscular route of application. As well as collecting pharmacokinetic data related to the toxicity trials, the goats were additionally observed for any signs of acute toxicity, of which none were seen at a total dose of 4 mg/kg for any of the compounds tested. Thereafter, tissue and organ samples were collected from each goat and prepared for histological examination.

Having concluded that DB 75, DB 867 and DB 1192 were considered safe in goats, they were furthermore investigated within an experimentally infected *Trypanosoma evansi* goat model. The efficacy of these compounds was examined at doses of 2.5 mg/kg and 1.25 mg/kg. Diminazene was included as a positive control drug and was given as 5 mg/kg. After treatment of the infected goats, a five month follow up study was conducted to determine the curative potential of the compounds used. Curative efficacy was assessed using three types of detection tests; a parasitological test (Haematocrit Centrifugation Technique), a serological test (CATT / *T. evansi*) and a molecular based test (PCR / *T. evansi*). At the end of the follow up efficacy trial, it was seen that DB 75 was able to effectively cure infected goats at a quarter of the dose (1.25 mg/kg) used by the positive control drug (Diminazene) and that DB 867 could cure infected goats at half the dose (2.5 mg/kg) used by that of diminazene. The third compound, DB 1192 was not able to treat goats at either the high or low doses tested and the infected goats relapsed, just two weeks post treatment.

Moreover, pharmacokinetic data was gathered from the efficacy study and the plasma samples collected were analysed using HPLC/MS-MS techniques, to determine the drug concentrations present in the goats over several time points. This enabled distinct pharmacokinetic profiles to be produced, revealing a clearer comprehension as to why DB 1192 failed to treat the experimentally infected goats. Drug concentrations obtained in plasma samples for DB 75 and DB 867 were found to be well within the therapeutic range. Several pharmacokinetic parameters were calculated to provide a better understanding of diamidines within goats. Furthermore, the long half lives of DB 75 and DB 867 may indicate drug accumulation within tissues, whilst the kinetoplast, nucleus and acidocalcisomes were identified as structures within trypanosomes where these diamidines accumulate, based on preliminary fluorescent experiments.

In conclusion, two novel diamidine compounds (DB 75 and DB 867) have been identified as potential clinical candidates against *Trypanosoma evansi* infection.

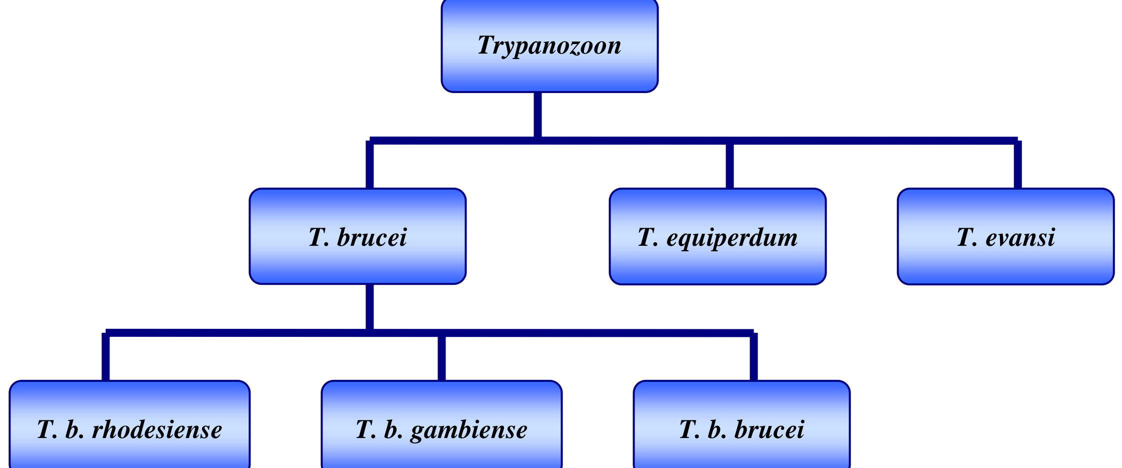
General Introduction

Trypanosomes

Within the order Kinetoplastida (family Trypanosomatidae), belong trypanosomes, haemoflagellated protozoan parasites characterised by one nucleus and one flagellum, either free or attached to the parasites body by means of an undulating membrane. They also usually contain a small, compact kinetoplast, a disc-shaped, DNA-containing organelle, situated within a large mitochondrion (*Brun et al., 1998*). Kinetoplast DNA is arranged into a network of linked circles, grouped into minicircles and maxicircles. Within the kinetoplast network, there are around 20’000 minicircles and 20-50 maxicircles.

A certain collection of salivarian trypanosomes can be further classified into the *Trypanozoon* group (*Figure 1.1*). Within this subgenus, there are three major trypanosome species; *Trypanosoma brucei*, *T. equiperdum* and *T. evansi*.

Figure 1.1. Taxonomic classification of the *Trypanozoon* group of salivarian trypanosomes.



T. brucei can be additionally divided into three subspecies, of which *T. brucei rhodesiense* and *T. b. gambiense* are the causative agents of the respective, acute form (found in Eastern Africa) and the chronic form (found in Western and Central Africa) of the debilitating sleeping sickness disease, Human African Trypanosomiasis (HAT). *T. b. brucei* is not human pathogenic and together with *T. congolense* (belonging to the subgenus, *Nannomonas*) and *T. vivax* (belonging to the *Duttonella* subgenus, which is also found in South America), is associated with trypanosomiasis within domestic animals and livestock,

causing a disease called Nagana. *T. equiperdum* appears to infect only equids (horses, donkeys and mules), causing a veneral disease called Dourine. *T. evansi* on the other hand, has the widest distribution of all species of trypanosomes and the greatest range of mammalian hosts (*Hoare, 1972*), making it one of the most economically important protozoan diseases present in the veterinary world today.

All these trypanosome species are morphologically indistinguishable from one another (except *T. congolense* and *T. vivax*), have a size range of 15-55 µm and typically live in the blood, lymph and tissues of their hosts. Bloodstream forms of trypanosomes are covered by a protective surface coat consisting of variant surface glycoproteins (VSG) linked in turn to the plasma membrane surface by means of glycosyl-phosphatidyl-inositol (GPI) anchors. This surface coat has the ability to undergo antigenic variation, whereby only one (out of a thousand) VSG gene is expressed at any one time (*Vickerman, 1985*). The trypanosomes can then spontaneously switch and express a different VSG gene, hence enabling an entirely different surface coat to be produced. Antibodies, previously mounted by an immune response, will not be able to recognise this newly expressed VSG gene and thus the trypanosome escapes detection. By the time the immune system has created new antibodies against this other VSG gene, the trypanosome has switched again, begun expressing an alternative VSG gene and as a consequence, can produce a persistent (chronic) infection. It is this special functioning coat, which helps trypanosomes evade the immune system and maintain their survival within hosts.

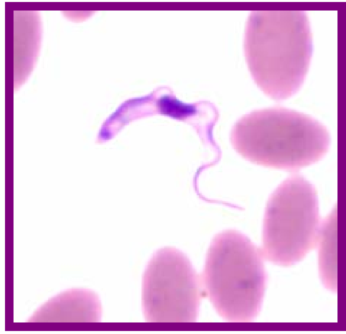
With the exception of *T. equiperdum* and *T. evansi*, which possesses only minicircles and no maxicircles within its kinetoplast, the majority of trypanosome species also undergo a developmental phase in insect vectors, such as the tsetse fly (*Vickerman, 1985*). In the host, short stumpy forms are the so-called non-dividing state and must be taken up by a feeding vector to ensure their continued survival and transformation into procyclic forms, where the protective VSG coat is lost. It is within the insect vector that trypanosomes undergo a chain of events involving differentiation, multiplication and biochemical alterations, such as swapping their energy metabolism from glucose (in bloodstream forms) to proline (in procyclic forms), before migrating to the salivary glands, where they progress into infective metacyclic forms by re-gaining their VSG coat and are then ready to be inoculated into a new host during the next bloodmeal. Once they enter their new host, these metacyclic forms differentiate into rapidly-dividing, long slender bloodstream forms, allowing the life cycle to complete itself, before short stumpy forms are then proliferated and the next insect vector is awaited

(*Vickerman, 1985*). Although multiplication generally occurs via binary fission, genetic exchange has been shown to occur in insect forms (*Jenni et al., 1986*).

Trypanosoma evansi

Trypanosoma evansi, a haemoflagellated parasite (*Figure 1.2*), is the causative agent of a progressive (and ultimately fatal) wasting disease called Surra. Other terms for this condition include "Mal de Caderas" in South America, "El debab" in the Middle East and Africa and "Gufar" in Sudan (*El Rayah et al., 1999*; *Giardina et al., 2003*). Surra is extensively distributed geographically, has a wide range of mammalian hosts and results in great economical loss in areas of Africa, Asia and South America as thousands of animals die each year from this infection.

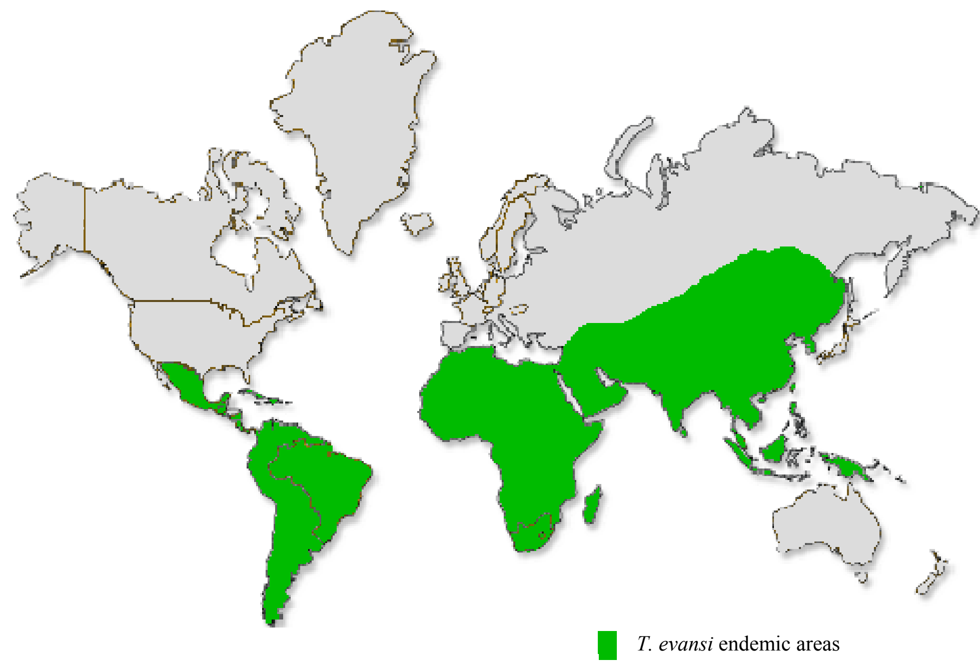
Figure 1.2. *Trypanosoma evansi* isolated from the blood of an infected camel (Giemsa stain).



Trypanosoma evansi is pathogenic in most domestic animals and some wild animals (*Lun et al., 2004*; *Zhou et al., 2004*). Horses, mules, donkeys, camels, llamas, deer, cattle, buffalo, cats and dogs are generally affected; yet asymptomatic, mild or chronic infection has also been seen in sheep, goats, elephants and pigs. However, the main host species varies according to geographical location: Camels are most often affected in the Middle East and Africa, horses in South America and horses, mules, buffalo and deer in China. Surra in Southeast Asia is mostly seen in horses, cattle and buffalo (*Holland et al., 2004*). In Central and South America, capybara and vampire bats act as reservoir hosts. Vampire bats even have the potential to operate as disease vectors (*Davila and Silva, 2000*). The endemic geographic distribution of this disease (*Figure 1.3*) ranges from China, the Philippines and parts of Indonesia, across Southeast Asia and parts of the former U.S.S.R, into the Indian

subcontinent, the Middle East and Africa, until it reaches Central and South America (*OIE, 2004*).

Figure 1.3. Geographical distribution of endemic areas of *Trypanosoma evansi* infection.



The severity of the disease varies according to the strain of *T. evansi* in question and host factors like general health and stress of the animal (*Kaminsky et al., 1997*). Symptoms of the disease include anaemia, recurrent fever and oedema with muscular weakness. There is usually a loss of appetite and a reluctance to move, the coat condition of the animal worsens and pregnant females can abort their foetuses (*Gutierrez et al., 2005*). Without effective treatment, animals infected with *T. evansi* face a fatal outcome.

Disease outbreaks tend to be associated with movement of infected animals into disease-free areas or susceptible animals into highly endemic regions. Morbidity can be 50 - 70 % in some outbreaks with a similar value for mortality. In China, the average mortality rate is 41 % in horses and 28 % in cattle and water buffalo (*Brun and Lun, 1994*). In Sudan, disease prevalence in camels is said to be reported at 20 %, which unsurprisingly increases during severe outbreaks (*Rae et al., 1989*). Few studies have been done on disease prevalence in endemic regions and consequently little is known about exact figures, but they are often thought to be much higher than actually described.

Transmission

Unlike other trypanosome species, *T. evansi* is not restricted to Africa alone, since transmission is mechanically performed by biting flies in the genera *Tabanus*, *Stomoxys* and *Lyperosia*, yet species of *Tabanus* (*Figure 1.4*) tend to be the most significant vectors, with only the females being haematophagous (*Luckins, 1992*).

Figure 1.4. One of the vectors (*Tabanus sp*) responsible for the mechanical transmission of *T. evansi*.

(Source: Bruce Marlin, Cirrus Digital Imaging, 2005)

Successful transmission depends on the time taken for an infected vector to find a new host, as the viable trypanosomes remain within the mouthparts of the flies only and are therefore susceptible to rapid dessication. No intermediate hosts are present and there is no insect developmental cycle available in *T. evansi* (*Foil, 1989*). Transmission in milk and during coitus has been reported and carnivores may pick up infection after feeding on infected meat (*Herrera et al., 2004*).

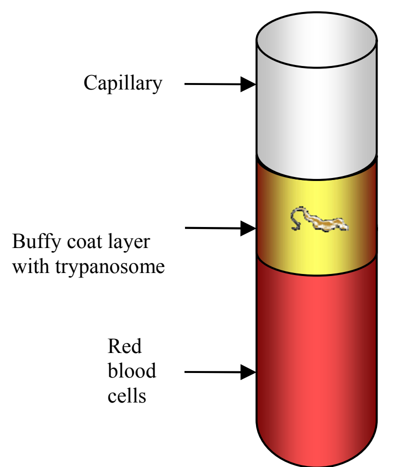
T. evansi has never really been human pathogenic, as shown by E. Brumpt himself, when he infected himself in 1902 with blood from the jugular vein of a dromedary camel, rich in *T. evansi*. He experienced no symptoms as a result of this injection. In summer, 1999, a patient in Colombo, Sri Lanka, was considered to have a *T. evansi* infection after having been in direct contact with cattle. The parasite was later identified as belonging to the *T. evansi* group, yet the trypanosomes disappeared from the patient's blood after a year. Recently, in the autumn of 2004, in the Indian state of Maharashtra, a male (also in close contact with cattle) contracted the first official human case of *T. evansi* infection (*Joshi et al., 2005*). Then in January, 2005, a female patient, from West Bengal state in India, actually died from a *T. evansi* infection, making her the second human case of this parasite. It was later identified that patients missing the typical trypanolytic factor (apolipoprotein L1) usually found in human

serum, resulted in their susceptibility to this infection (*Vanhollebeke et al., 2006*). It is still too early to consider *T. evansi* as a new zoonosis; however, should this be the case, major implications could occur for future chemotherapeutic research against this parasite (*Touratier, personal communication, 2005*).

Diagnosis

It is vital to be able to diagnose *T. evansi* infection properly for effective control purposes, as disease symptoms are generally too vague. Several techniques are available depending on the type of diagnostic approach followed. For parasitological tests, a thick or thin wet blood smear can be performed and examined under light microscopy. Positive results are achieved upon detection and observation of the parasite in the blood (*OIE, 2004*). For infections with a low parasitaemia, concentration techniques can be carried out, where the trypanosomes are concentrated into a specific region and then observed under light microscopy. The haematocrit centrifugation (HCT or Woo) technique (*Figure 1.5*) involves filling heparinised capillary tubes with the blood sample, centrifuging the capillaries and then searching for viable parasites in the so-called buffy coat layer, found between the red blood cells and the plasma (*Woo, 1970*).

Figure 1.5. Diagrammatic representation of the HCT for parasitological diagnosis of *T. evansi*.



A second concentration method, the mini-AECT (Anion Exchange Centrifugation Technique) involves the blood sample passing through a DEAE-cellulose column, which separates trypanosomes from red blood cells, based on charge. The highly negatively-charged red blood cells stick to the column, whilst the trypanosomes pass through the column and are collected as eluate at the other end. This eluate can then be centrifuged and examined for the presence of trypanosomes (*Lumsden et al., 1979*). A further parasitological test includes animal inoculation, where the suspected blood sample is inoculated into small rodents (usually rats or mice) and in positive cases, a parasitaemia will establish itself. The small rodents are checked regularly for the presence of *T. evansi* parasites via tail blood examination.

Another approach is to use serological tests, such as the indirect fluorescent antibody test (IFAT) or the enzyme-linked immunosorbent assay (ELISA), where specific antigens against the parasite are previously fixed to a slide (IFAT) or a microtitre plate (ELISA). The test serum is placed on the slide/plate and then washed off. If antibodies against the parasite are present in the test serum, they stick to the respective antigens and are left on the slide/plate after washing. An enzyme labelled conjugate is then applied to the slide/plate. A positive reaction binds the conjugate, causing it to fluoresce, which is then detected when examined under UV light in a microscope or fluorescent plate reader (*OIE, 2005*). Recently, a card agglutination test for trypanosomiasis (CATT) was designed to detect antibodies against *Trypanosoma evansi* (CATT/*T. evansi*) (*Bajyana Songa and Hamers, 1988*; *Diall et al., 1994*; *Gutierrez et al., 2000*); the first CATT test was produced for *T. b. gambiense* detection (*Magnus et al, 1978*). Here, test serum is diluted and placed onto the card within the circles. The reagent containing a specific antigen is placed on top, mixed carefully and the card is allowed to rotate on a motorised rotating machine (*Figure 1.6*). Samples which agglutinate are considered positive for infection. However, the presence of antibodies does not determine whether an infection is current or past and therefore serological tests are unable to detect new infections, where antibodies might not have developed yet.

Figure 1.6. The CATT/*T. evansi* test used for the serological diagnosis of *T. evansi* infection.

A final approach may be to use molecular techniques based on specifically designed sequences, where detection of parasite DNA indicates the active infection and presence of the parasite in the sample. It is believed that parasite DNA will not persist within the host for long, after all viable parasites have been eliminated. Molecular techniques, of which the most well known is the polymerase chain reaction (PCR), tend to be highly specific and sensitive and may constitute as the gold standard test for many infections. The PCR process allows a

small amount or a specific region of DNA to be amplified, using precise temperature conditions and ingredients like primers, which read the specific region, a DNA polymerase, which can synthesise a copy of the DNA region and deoxynucleotide triphosphates (dNTPs), which build up the new DNA copy (*Holland et al., 2001*). However, most molecular tests usually require well-equipped laboratories, expensive reagents and well-trained personnel. Due to such features, these techniques remain primarily as research tools and are not currently utilised as routine diagnostic tests.

Control

Vector control has demonstrated an effective measure for controlling the tsetse fly situation with HAT. Through constant maintenance of cleverly-designed tsetse fly traps and insecticidal spraying regimens, it is possible to significantly reduce the number of tsetse flies in a given region. The tsetse fly population was actually successfully eradicated on the island of Zanzibar in 1998, through the release of sterile male tsetse flies, which competed with the natural male population and resulted in infertile female flies unable to lay larvae (*Vreysen et al, 2002*). This method is known as the sterile insect technique (SIT). Whether this approach will be as successful in the expanded area of mainland Africa, in connection with the geographical tsetse fly habitat belt, is not yet evident.

Unfortunately, vector control for the prevention of *T. evansi* infection is not considered a viable option, since transmission occurs mechanically and potentially any blood-sucking insect has the ability to transmit this infection. Based on its vast area of global endemicity, vector control against *T. evansi* infection would also result in an unnecessary economical drain on several countries involved.

Control measures for protozoan diseases, such as Surra, therefore tend to rely on either treatment or prophylaxis. Treatment usually involves the use of anti-parasitic drugs, whereas prophylaxis can be accomplished by either drugs or vaccination. Whether “prevention is better than cure” depends on the parasite being dealt with, the type of drugs available, the occurrence and severity of the disease and the economics obtainable in such a situation (*Ryley, 1982*).

Treatment

Chemotherapeutic treatment against *T. evansi* infection is currently dependent on four main drugs, namely Germaine® (seamen), Berenil® (diminazene aceturate), Trypacide® (quinapyramine sulphate) and MelCy® (cymelarsan) (*Zhang et al., 1992*). While the first three have been utilised for more than 50 years (*Kaminsky and Zweygarth, 1989*), cymelarsan, belonging to the family of melaminophenyl arsenicals, was pharmaceutically developed less than twenty years ago (*Williamson, 1970; Raynaud et al., 1989; Tuntasuvan, 2003*).

Suramin (Germanin®, Bayer in Germany) was originally synthesised as a dye in 1916. This naphthalene compound (*Figure 1.7*) binds strongly to plasma proteins and low-density lipoproteins (LDL), followed by a subsequent slow release mechanism. Due to the molecule's charge and large size, diffusion and/or endocytosis are believed to be the key methods of uptake into the parasite. Its mode of action is not fully understood, yet anionic interactions with enzymes, in particular those associated with glycolysis in trypanosomes, would enable inhibition of reverse transcriptase and receptor mediated uptake of low-density lipoproteins by trypanosomes to occur (*Cheson et al., 1987*; *Vansterkenburg et al., 1993*; *Denise and Barrett, 2001*). Suramin is principally used for the early treatment of East African *T. b. rhodesiense* sleeping sickness in humans, as well as for *T. evansi* infections in animals.

Figure 1.7. Chemical structure of suramin, a naphthalene compound.

Diminazene aceturate (Berenil®, Sigma in USA) is a triazene molecule, belonging to the aromatic diamidines, of which pentamidine is the best known example, due to its use against the early stages of the chronic *T. b. gambiense* form in humans, along with early stage visceral leishmaniasis and *Pneumocystis carinii* pneumonial infections in HIV-infected individuals. Diminazene (*Figure 1.8*) was originally discovered in 1944, having been developed based on compounds similar to amino-quinaldines, with good anti-trypanosomal properties (*Peregrine and Mamman, 1993*). It is actively taken up through a nucleoside P2

transporter, coded by the *TbAT1* gene, in trypanosomes (*Mäser et al, 1999*). This transporter is vital in the uptake of adenosine and adenine within trypanosomes, allowing them to salvage purines and nucleoside bases. Its mode of action is believed to centre on nucleic acid binding, especially at AT-rich regions of the minor groove in DNA (*Wilson et al., 2005*).

Figure 1.8. Chemical structure of diminazene aceturate, an aromatic diamidine.

Cymelarsan (MelCy®, Rhône Mérieux in France) is a member of the melaminophenyl arsenical family and thus related to melarsoprol, the first line drug for the second stage treatment of human African Trypanosomiasis. The first organic arsenical drug, Atoxyl®, appeared in 1905 for the treatment of syphilis. It included slight efficacy against trypanosomes, but its toxicity was unbearable (*Williamson, 1970*). Thereafter, thousands of similar compounds were then synthesised, whilst trying to reduce the toxic effects of this chemical family. Finally in the 1940s, melarsoprol was produced, demonstrating a breakthrough in the treatment of the late stage of human African sleeping sickness (*Friedheim, 1949*). Following this success, cymelarsan (*Figure 1.9*) arrived on the scene, as a prodrug called melarsenoxide cysteamine, requiring two cysteamine molecules to be released to produce its activity.

Figure 1.9. Chemical structure of cymelarsan, a melaminophenyl arsenical.

Cymelarsan is a highly active drug against *T. evansi* infection, yet its downfalls revolve around its toxicity, expensive market price and global unavailability. It is taken up into trypanosomes with the same nucleoside P2 transporter as that seen for diminazene (*Carter and Fairlamb, 1993*), whilst its mode of action involves a high affinity for

intracellular thiols, in particular trypanothione, which is necessary for the redox balance of trypanosomes.

Quinapyramine sulphate (Trypacide®, May & Baker in Nigeria) is a quinoline pyramidine, used to treat trypanosomiasis in cattle from the 1950s until the 1970s. Resistance to this compound built up rapidly and coupled with severe toxicity, production ceased in 1976 and quinapyramine was removed from the market. Nevertheless, in 1984 it was reintroduced to the market for use solely in camels (*Ndoutamia et al., 1993*). Even so, several African countries are still using quinapyramine for the treatment of livestock (including cattle) despite the problems experienced 30 years ago. Quinapyramine (*Figure 1.10*) is thought to act indirectly by inhibiting protein synthesis through the displacement of magnesium ions and polyamines from ribosomes. Uptake is probably through the P2 transporter, similar to diminazene and cymelarsan.

Figure 1.10. Chemical structure of quinapyramine, a quinoline pyramidine.

Standard drugs

Several problems are associated with the current standard drugs available for the treatment of *T. evansi* infection. Resistance to drugs tends to occur in situations, where the drugs available are old and few and hence are repeatedly used over a long period of time. Together with possible under-dosing, where inadequate or erroneous applications of subcurative doses of the drugs are given (especially under field conditions) (*Bacchi, 1993*), natural selection pressures instantly favour less drug-sensitive trypanosomes. Drug resistance in trypanosomes arises through either the loss or alteration of certain transporter proteins, implying a decrease in the amount of drug entering the parasite, or through increased expression of efflux pumps, which then expels the drug at a faster rate from the parasite, than it can actually enter the cell (*Mäser and Kaminsky, 1997*).

With the rising appearance of resistance to these drugs, their effective use is severely threatened. For example, *Zhang et al.* (*1993*) induced resistance in laboratory mice resulting in *T. evansi* resistance to suramin, diminazene and cymelarsan, indicating that inappropriate use of these drugs can easily lead to resistance in the field. *Zhou et al.* (*2004*) tested various *T. evansi* isolates and found differences in their levels of sensitivity to suramin and quinapyramine, with some being highly sensitive and others showing complete drug resistance. No cross-resistance was observed between these two drugs. Similarly, *El Rayah et al.* (*1999*) demonstrated suramin and quinapyramine resistance in sixteen *T. evansi* isolates from Sudan. In addition, several studies have already shown the possible cross resistance between diamidine drugs, such as diminazene, and melaminophenyl arsenical compounds, such as cymelarsan (*Zhang et al., 1993*; *Ross and Barns, 1996*). Since the P2 transporter is involved in the active drug uptake of diamidine compounds and melaminophenyl arsenicals, loss of this P2 transporter would render that organism resistant to such compounds (*Carter and Fairlamb, 1993*; *Carter et al., 1995*; *Barrett et al., 1995*).

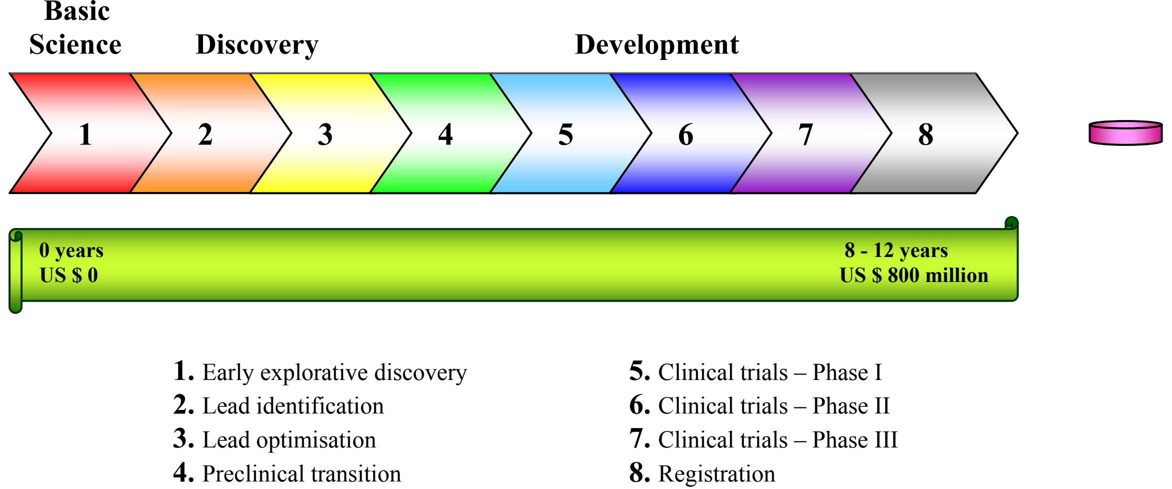
Many of these drugs have already lost their ability to cure infected animals efficiently; some have toxicity associated with their use in certain species or are just too costly for the poorer communities within the world to afford. If there were even an alternative, the circumstances might not look so dire, yet alternatives are not currently accessible, due to a sparse drug repertoire. Furthermore, for those communities that can afford the more costly standard drugs, treatment of their infected animals is not made easier, thanks to the fact that some drugs are simply not obtainable within that country's market. With such limited availability, soaring expenses and emerging drug resistance, many *T. evansi* infections are reaching epidemic proportions in regions, where the loss of livestock and expensive, prestigious animals can have staggering repercussions and consequences, both for the individuals and the communities involved (*Touratier, personal communication, 2006*).

In the case of Surra, drugs containing antiparasitic properties were usually administered once an infection had already established itself, in the hope that such activity would render the parasite non-viable and the infection would disperse (*Davila and Silva, 2000*; *Seidl et al., 2001*). However, this tactic is now failing miserably and the pressing need for new, alternative chemotherapeutic agents is becoming critical (*Touratier, 2000*; *Reid, 2002*). Moreover, the prospect of a vaccination against *T. evansi* infection appears non-existent, for which a large proportion of blame can be placed upon the mystifying antigenic variability of trypanosomes in general.

Drug discovery

Drug research and development for animal diseases is a similar process to that involved for human diseases. There are some key differences however between the two, which should be taken into account when regarding the clinical trials and costs of drug discovery for animal diseases. Nevertheless, drug discovery for human diseases will be discussed initially to enable these differences to be adequately highlighted. To discover a new drug is a timely, expensive and immense process, which involves several different stages of research, together with numerous developmental phases, followed by the final regulatory affairs section deciding the overall outcome of the new drug (*Figure 1.11*). The overall estimated time involved in discovering, developing and registering a new drug is between eight to twelve years. Often the disadvantages of a new drug outweigh the beneficial risks, so that many potential drug candidates are "dropped" during the discovery process as the investment costs associated with them are clearly too high. Figures currently demonstrate that on average, US$ 800 million need to be invested, from start to finish, for the complete development of merely one new drug. This figure incorporates all the drug failures too. In addition to this, production success rates are poor, as only 1 - 2 drugs actually reach registration out of every 15'000 compounds originally synthesised (*Nwaka and Hudson, 2006*).

Figure 1.11. Drug pipeline showing the various stages, time and costs involved in producing a new drug against human diseases.



Naturally, large pharmaceutical companies tend to be in possession of such financial sums and therefore are the industrial leaders in manufacturing new drugs. However, no business is prepared to invest millions to produce a drug, which provides no safe assurance, that a financial return will be made. Such attitudes would only result in the demise of these businesses. To add to this conundrum, the rules and regulations required to be met by new drug candidate molecules have been tightened and strict health and safety guidelines must now be met in order to produce new drugs for our markets. Developing and producing drugs was easier 20 - 30 years ago, when these guidelines were not as strict, hence many of the drugs found on our market today would never have been passed and registered according to the present day guidelines.

Good examples can be seen in tropical and infectious diseases, where the majority of drugs available for treating parasitic, viral or bacterial infections, generally found in tropical climates, are so toxic that the drugs themselves cause fatalities. Melarsoprol, mentioned earlier as the drug of choice for late stage African trypanosomiasis, is associated with an adverse post treatment reactive encephalopathy, occurring in 5 - 10 % of all patients treated. Of this 5 - 10 %, a staggering 50 - 70 % of them die as a result of this adverse effect (*Pepin and Milord, 1991*). Nonetheless, patients suffering from this disease have nothing to lose, especially when faced with a 100 % mortality rate if the infection goes untreated.

Although the choices are bleak for those suffering from or at risk of contracting tropical and infectious diseases, the conditions appear more desperate when faced with the possibility that the only remaining (albeit toxic) drugs no longer work against infection, coupled with the fact that such powerful and effective drugs are no longer coming out of the drug discovery process, as a direct result of the new rules and regulations. The harsh reality continues whereby those most in need of such drugs include some of the poorest regions in the world, hence no pharmaceutical company is interested in investing millions to produce drugs that cannot provide a decent financial return.

Therefore to summarise, the situation appears hopeless for all involved – the pharmaceutical industry cannot produce drugs with no financial return, otherwise their businesses will suffer and the success rate of new drug candidates decreases further, as less candidates will pass the new health and safety regulations, providing larger financial losses for pharmaceutical companies and consequently resulting in fewer new drugs being introduced onto our markets. This in turn condemns those requiring the drugs, be it humans or animals, many of whom will not be able to pay for the new drugs anyway. With this sorry

sight, pharmaceutical companies retracted from investigating new drugs for tropical medicine and for a long time, nothing was done to improve the situation.

At the beginning of the 21st century, this gap was bridged with the manifestation of public-private partnerships (PPPs), consortia made up of various governmental research departments and institutes (the public half), along with several small pharmaceutical entities (the private half), all with the general goal to improve the drug situation against parasitic diseases occurring in developing countries. New drugs were to be discovered through *in vitro* and *in vivo* screening techniques, using the facilities available in research institutes, followed by the private sectors undertaking the more costly, preclinical (ADME and toxicity) and clinical trials. Overall, new, safer and cheaper compounds, featuring high anti-parasitic activity, were aimed to be discovered.

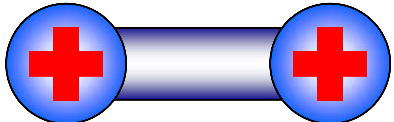
In the case of animal health, several studies need to be presented in order to pass a new drug onto the veterinary market as a safe and reliable product. Such tests include *in vitro* and *in vivo* experiments with laboratory animals. Studies performed in a target species investigating tolerance, reproductive features, as well as the class of animal and specific breeds targeted, along with various field investigations and environmental safety assessments must be carried out. A range of toxicity trials including aspects of mutagenicity, reproduction, resistance studies and user safety must be dealt with. A large proportion of the regulations focus on the clinical phases, in particular on drug residue, whereby the total metabolism within the target animal must be demonstrated and understood, in addition to the comparative metabolism within rodents, tissue residue depletion analysis and method validation. This is critical for any drugs wishing to target livestock or agricultural animals, since meat, milk and other dairy products will be consumed by humans and any acute or chronic toxic responses need to be fully examined before a new drug can be approved. New veterinary drugs must be approved by the Centre for Veterinary Medicine (CVM), which is a division of the United States of America, food and drug administration (FDA), before they can enter the global market.

Diamidines

With the introduction of novel aromatic diamidines (dicationic molecules), a new class of anti-trypanosomal drugs was revealed (*Francesconi et al., 1999*; *Wilson et al., 2005*). Their principle activity is based on the two functional amidine groups separated by a spacer region, the distance of which is important for optimal compound activity (*Figure 1.12*). These

chemicals bind to the minor-groove of DNA at AT-rich sites. Their interaction with the DNA minor groove has been examined at a molecular level, using biophysical techniques and X-ray crystallography, concluding that these compounds apply their biological activity by primarily binding to DNA and then inhibiting one or more of the DNA dependent enzymes (such as topoisomerases or nucleases) or by directly impeding the transcription process (*Wilson et al., 2005*). The selective binding to kinetoplastic DNA has also been shown to play a vital role in the action of aromatic diamidines against pathogens (*Francesconi et al., 1999*).

Figure 1.12. Aromatic diamidines based on the two cationic moieties separated by the spacer region.



The first antiprotozoan diamidine was called synthalin, a diguanidine originally produced as a synthetic analogue of insulin. It was tested on trypanosome infections with the theory that induced hypoglycaemia could control these infections in mammals (*Von Jansćo and Von Jansćo, 1935*). In 1937, synthalin was discovered to have similar activity against trypanosomes *in vitro* as trivalent arsenicals (*King, 1937*). From this, further development and testing lead to a large number of related compounds being produced. It was then that aromatic diamidines, such as pentamidine, were noticed as highly effective in treating first stage (and early second stage) trypanosomiasis (*Lourie and Yorke, 1939*), as well as other infections, such as leishmaniasis and *Pneumocystis carinii* pneumonia.

With regard to human African Trypanosomiasis, several diamidine compounds have shown outstanding success. The compound DB 289, a prodrug of DB 75, is currently in phase III clinical trials. Although only sub-curative levels cross the blood brain barrier, it looks promising as a safe and effective oral drug against the early stage of the disease, not to mention more cost-efficient (*Legros, 2002*). Another excellent candidate is DB 844, a prodrug of DB 820, which does possess the ability to cross the blood brain barrier and could therefore be an alternative to melarsoprol for treating the second stage of sleeping sickness.

Based on such hopeful results observed with diamidines against the *T. brucei* species, considerations were made whether a similar level of success could be achieved with *T. evansi*. An alternative drug for *T. evansi* would produce a major benefit to the present market, which is currently hindered by the emergence of drug-resistant strains, old and ineffective drugs,

severe drug toxicity and expensive alternatives, leading to great economic loss for those who depend entirely on their domestic animals for income. A cheap, safe and efficient drug, which can easily be synthesised, is available for large-scale production and has a reduced potential for future drug resistance is desperately sought after. All these aspects are vital to ensure the successful control of *T. evansi* infection, where the most reliable method of disease control still remains the administration of anti-trypanocidal drugs.

Aims and objectives

The main aim of this thesis is to discover and develop a potential clinical candidate compound (or compounds), for the future drug development against *Trypanosoma evansi* infection. By means of *in vitro* and *in vivo* drug screening, the most active compounds can be selected for and tested in a higher animal model before further clinical trials are undertaken. Through preliminary toxicity tests and a "proof of concept" pilot study, additional data on diamidines can be attained and all-important drug levels can be determined and pharmacokinetic parameters can be calculated. A better understanding of drug metabolism within higher animals is expected to be gained. Together with a clearer insight into the exact mode of action and uptake processes involved in diamidines, it will be possible to establish how these compounds are taken up by trypanosomes and where they exert their activity.

Precise objectives include:

- To establish the drug profile of a global panel of *Trypanosoma evansi* and *Trypanosoma equiperdum* strains to be used for future drug screening studies

- To test various diamidine compounds and determine *in vitro* activity against *Trypanosoma evansi* strains to identify molecules for animal model experiments

- To analyse the most active diamidine compounds and investigate their *in vivo* toxicity and efficacy within a mouse model

- To perform a pharmacokinetic study and gain vital information over such parameters for active diamidine compounds using an experimentally infected *Trypanosoma evansi* goat model

- To undertake a "proof of concept" pilot trial to verify the treatment efficiency of active diamidine compounds within an experimentally infected *Trypanosoma evansi* goat model

- To explore the mode of action and mechanism of uptake used by these active diamidine compounds using fluorescent techniques

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Establishment of a panel of reference *Trypanosoma evansi* and *Trypanosoma equiperdum* strains for drug screening

Kirsten Gillingwater[1], Philippe Büscher[2] and Reto Brun[1]

[1]Parasite Chemotherapy, Swiss Tropical Institute, Socinstrasse 57, 4002 Basel, Switzerland
[2]Department of Parasitology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium

Accepted for publication in

Veterinary Parasitology

Abstract

The animal pathogenic protozoan, *Trypanosoma evansi*, leads to a wasting disease in equines, cattle and camels, commonly known as Surra. It is extensively distributed geographically with a wide range of mammalian hosts and causes great economical loss. *Trypanosoma equiperdum* causes a venereal disease called Dourine in horses and donkeys. Chemotherapy appears to be the most effective form of control for *T. evansi*, whereas infections caused by *T. equiperdum* are considered incurable. Due to emerging drug resistance, efficient control of *T. evansi* is severely threatened, emphasising the urgent need to find new alternative drugs.

A drug profile for a panel of *T. evansi* and *T. equiperdum* strains has been established for the four standard drugs currently used in treatment. The ^{3}H-hypoxanthine incorporation assay was used to obtain 50 % inhibitory concentration (IC_{50}) values for each standard drug against the various strains. The results indicate the presence (and in some cases, the emergence) of drug resistance in several strains. This panel of characterised strains with known drug sensitivities and resistances will be of great value for the screening of new active compounds, in comparison with the four standard drugs currently available.

Keywords: *Trypanosoma evansi*; *Trypanosoma equiperdum*; chemotherapy; drug resistance; drug screening.

Introduction

Trypanosoma evansi is a flagellated animal pathogenic protozoan parasite, where infection leads to a wasting disease called Surra. This disease has a wide geographical distribution and range of mammalian hosts, causing great economical loss in areas of Africa, Asia and South America as thousands of animals die each year resulting from infection (*Giardina et al., 2003*). *T. evansi* is pathogenic in most domestic animals and some wild animals (*Lun et al., 2004*; *Zhou et al., 2004*). However, the main host species varies according to geographical location: Camels are most often affected in the Middle East and Africa, horses in South America and horses, mules, buffalo and deer in China. The severity of the disease

varies according to the strain of *T. evansi* in question and host factors like general health and stress of the animal (*Brun et al., 1998*).

Unlike other trypanosome species (such as *Trypanosoma brucei brucei* and *Trypanosoma congolense*), *T. evansi* is not restricted to Africa alone, since transmission is mechanically performed by biting flies of the genera *Tabanus*, *Stomoxys* and *Lyperosia*. Successful transmission depends on the time taken for a contaminated vector to find a new host, as the viable trypanosomes remain within the mouthparts of the flies only and are therefore susceptible to rapid dessication. No intermediate hosts are present and there is no insect developmental cycle available in *T. evansi* (*Foil, 1989*).

Although *T. evansi* and *T. equiperdum* are closely related, they remain classified as two distinct species (*Hoare, 1972*; *Claes et al., 2003*; *Claes et al., 2006*). *T. equiperdum* causes a venereal disease called Dourine in horses and donkeys and is morphologically indistinguishable to other *Trypanozoon* species (*Verducci et al., 1989*; *Brun et al., 1998*). Disease transmission is thought to occur during coitus as no arthropod vector has been discovered for this species. Dourine is found in Africa, Asia, Southern and Eastern Europe, Russia and Mexico (*Davila and Silva, 2000*).

Both *T. evansi* and *T. equiperdum* infections are fatal if left untreated. *T. equiperdum* infections are considered incurable in terms of chemotherapy, where administered drugs can reach parasites within the blood, yet cannot necessarily access parasites hidden in certain tissues (*OIE, Surra fact-sheet, 2004* (www.oie.int/eng.htm); *OIE homepage, 2005* (www.oie/int/eng/en_index.htm); *Claes et al., 2005*), while treatment for *T. evansi* infection is dependent on four drugs, namely suramin, diminazene aceturate, quinapyramine and cymelarsan (*Zhang et al., 1992*; *Brun and Lun, 1994*). While the first three have been utilised for more than 50 years, cymelarsan, belonging to the family of melaminophenyl arsenicals, was pharmaceutically developed less than twenty years ago (*Williamson, 1970*; *Zelleke et al., 1989*; *Raynaud et al., 1989*; *Tuntasuvan, 2003*). As drug resistance is emerging, severe drawbacks in controlling *T. evansi* are occurring, hence emphasising the urgency and necessity for finding new alternative drugs (*Kaminsky et al., 1989*; *De Koning, 2001*; *Suswam et al., 2001*). Inappropriate use of these drugs can easily lead to resistance in the field, as shown by *Zhang et al.* (*1993*), who induced resistance in laboratory mice resulting in *T. evansi* resistance to suramin, diminazene and cymelarsan. *Zhou et al.* (*2004*) tested various *T. evansi* isolates and found differences in their levels of sensitivity to suramin and quinapyramine, with some being highly sensitive and others showing complete drug

resistance. Similarly, *El Rayah et al.* (*1999*) demonstrated suramin and quinapyramine resistance in 16 *T. evansi* isolates from Sudan.

Since many of the hosts affected in these disease endemic countries represent expensive and prestigious animals, a new drug against these trypanosome infections would have a potentially high market value amongst the other antitrypanosomal agents. In order to aid drug discovery within this field, a panel of reference *T. evansi* and *T. equiperdum* strains was established and their sensitivity profiles in relation to existing drugs were determined.

Materials and Methods

Parasite strains

In total, eleven *Trypanosoma evansi* strains were included in this study (*Table 2.1*). The STIB 806K (kinetoplastic) strain was isolated by ZR Lun, from a water buffalo in China and was used as a reference strain in all experiments performed. Two African strains, STIB 780 and STIB 781, were received from the International Livestock Research Institute (ILRI) in Nairobi, Kenya. The remaining eight *T. evansi* strains were obtained from the cryobank of the Institute of Tropical Medicine (ITM) in Antwerp, Belgium.

Likewise, eleven *Trypanosoma equiperdum* strains were also used for this study (*Table 2.1*). All *T. equiperdum* strains were obtained from the ITM cryobank in Antwerp, Belgium, with the exception of STIB 818, which was collected by ZR Lun from China. The BoTat 1 (Bordeaux *Trypanosoma* antigen type 1) clone was derived from a *T. equiperdum* strain from the Institut Pasteur in Paris, France. The strain, originally isolated in 1924 from a horse in Morocco, was transferred to Bordeaux in 1961 and maintained in serial passages in mice until 1971, whereupon it was cloned and stored in liquid nitrogen. The BoTat 1.1 strain was designated as a reference strain in all experiments performed.

Unfortunately, the precise history for some of the strains is unknown, especially in the case of the AnTat 4.1 (Antwerp *Trypanosoma* antigen type 4) strain, the Hamburg strain and the SVP (Staatliches Veterinärmedizinisches Prüfungsinstitut) strain. Nevertheless, the Hamburg, SVP and Alfort strains are currently used as reference *T. equiperdum* strains for the diagnosis of dourine in Germany. Even though the majority of data for these strains are

unknown, this remains the most globally updated list of *T. equiperdum* strains (*Claes et al., 2005*).

Mice

All mice used were female NMRI, aged between 3 and 4 weeks and were maintained under standard animal housing facilities in air conditioned rooms (22 - 23 °C), with a relative humidity of 60 - 70 %. The mice were specific pathogen free (SPF) and were kept in standard Macrolon type II cages, with pelleted food and water *ad libitum*.

Standard trypanocidal drugs

Suramin (Germanin® from Bayer, Leverkusen, Germany), diminazene aceturate (D-7770, Sigma, St Louis, MO, USA), cymelarsan (MelCy®, Rhône Mérieux, Toulouse, France) and quinapyramine sulphate (Trypacide®, May & Baker, Lagos, Nigeria) were used as the standard drugs in this study.

Stock solutions and dilutions

A 10 mg amount of each drug was weighed out in powder form and dissolved in 1 ml of sterile distilled water, to provide a 10 mg/ml stock solution. These stock solutions were then stored frozen at -20 °C. From these stock solutions, further drug dilutions were made for use in the *in vitro* drug sensitivity assays, using culture medium as a solvent. Drug dilutions were made fresh on the day of each experimental assay procedure.

Culture medium

Bloodstream form trypanosomes were cultivated in Minimum Essential Medium (MEM) (powder, GIBCO/BRL, No: 11400-033) with Earle's salts, supplemented with 25 mM HEPES, 1 g/l additional glucose, 2.2 g/l $NaHCO_3$ and 10 ml/l MEM non-essential amino acids (50x concentration). The medium was then further supplemented by adding 1 % of a 2-mercaptoethanol stock (14 µl of 12 mM 2-mercaptoethanol was diluted in 10 ml of distilled water), 1 % of a stock consisting of 100 mM sodium pyruvate and 50 mM hypoxanthine and 15 % heat inactivated horse serum, according to *Baltz et al.* (*1985*). The complete medium is

called Baltz MEM (BMEM). For the *in vitro* drug sensitivity assays, the 50 mM hypoxanthine was not present in the complete BMEM medium used.

Radioactive hypoxanthine

Radioactively labelled (8-^{3}H) hypoxanthine (TRK74, Amersham Biosciences UK Limited, Buckinghamshire, UK) was used for the drug sensitivity assays.

Average survival in mice

Female NMRI mice (two mice per strain) were infected with the different *T. evansi* and *T. equiperdum* strains. As a control, the average survival day for each strain was calculated after infecting the mice with 10^4 parasites and then allowing the parasitaemia to establish itself. The development of parasitaemia was observed daily by using a tail blood examination technique. Once these average survival days were known, experimental trypanosome populations could be propagated successfully, through infecting two new NMRI mice (per strain) with a 10^4 parasite concentration and then collecting the trypanosomes at peak parasitaemia.

In vivo trypanosome propagation

Trypanosomes were collected from donor mice using a 1 ml syringe with a 25 gauge sterile needle containing 20 µl of heparin. Once the blood had been collected, it was placed into a 5 ml Bijou bottle and immediately placed on ice. Thereafter, the mouse blood was passed through a DEAE (diethylaminoethyl)-cellulose column, in order to separate the trypanosomes from the blood. The filtrate was collected in a 15 ml Falcon tube and left on ice.

In vitro drug sensitivity assay

Once the trypanosomes had been passed through the DEAE-cellulose column, the resulting suspension could be concentrated by centrifugation, resuspended in fresh culture medium and counted using a cell analyser system (CASY, Schaerfe System, Reutlingen, Germany). The ^{3}H-hypoxanthine incorporation assay was used to determine the drug sensitivity for all four standard drugs for each of the trypanosome strains tested. The exact

assay procedure is detailed in *Brun and Kunz* (*1989*) and has been modified slightly for use in this study.

Briefly, 50 µl of BMEM containing no hypoxanthine, were added to each well of a 96-well microtitre plate, except for the last four wells on the top row (where 100 µl were added instead to act as a negative control) and all the wells on the second row. The test compounds were applied at 75 µl volumes into the empty wells of this second row, according to the required starting concentration of each compound being tested. Thereafter, 25 µl were removed from this second row using a multi-channel pipette and mixed with the wells in the row beneath. Again 25 µl were removed from this third row, placed into the next row down and mixed several times. This step was repeated until the last row had been reached. The final 25 µl from the last row were discarded. This process created a 3-fold serial compound dilution down the microtitre plate, which enabled a compound range of 1000 - 1.37 ng/ml to be tested for suramin, diminazene and quinapyramine. The compound range tested for cymelarsan included 30 - 0.04 ng/ml. In the case of suramin, an additional compound range, starting at 30 µg/ml was applied. The trypanosome density was then adjusted to provide a $2x10^6$ / ml starting concentration. A volume of 50 µl of this trypanosome suspension was then added to all 96 wells, with the exception of the four negative controls in the top row, and the plates were then incubated in a humidified atmosphere at 37 °C and 5 % CO_2. After 24 hours incubation, the plates were removed and 20 µl of a 1 µCi solution of radioactive hypoxanthine mixed with BMEM (containing no hypoxanthine) were placed into each well. The plates were then returned to the incubator for a further 16 hours incubation under the same conditions. After a complete incubation time of 40 hours, the plates were removed from the incubator and the wells harvested using a 96-well harvester (1290-004 Betaplate™, Berthold Technologies (Schweiz) GmbH, Regensdorf, Switzerland), followed by a liquid scintillation counter (1205 Betaplate™, Berthold Technologies (Schweiz) GmbH, Regensdorf, Switzerland) to measure the radioactivity. All experiments were performed three to four times and in duplicate. The data obtained were further analysed by transferring them into a standard operating procedure template in a graphics programme (Microsoft Excel) for determination of IC_{50} values.

Results

In this study, a total of eleven *T. evansi* and eleven *T. equiperdum* strains were available for drug profiling. Out of these twenty-two strains, twenty were successfully propagated in mice and then tested with the ^{3}H-hypoxanthine incorporation assay to give IC_{50} values against the four standard drugs currently used for treatment. The average survival days for NMRI mice, when infected separately with either the *T. evansi* or *T. equiperdum* study strains is shown in *Table 2.1*.

Table 2.1. *Trypanosoma evansi* and *Trypanosoma equiperdum* strain information detailing the originating country, host and year isolated including the average survival days for NMRI mice infected separately with these study strains.

Strain	Country	Host	Year isolated	Average survival (days)
***T. evansi* strains**				
CAN 86/Brazil	Brazil	Dog	1986	8
Colombia	Colombia	Horse	1973	8
Kazakhstan	Kazakhstan	Bactrian camel	1995	8
Merzouga 56	Morocco	Dromedary camel	1998	--
MHRYD/Brazil	Brazil	Capybara	1986	--
Philippines	Philippines	Water buffalo	1996	6
RoTat 1.2	Indonesia	Water buffalo	1982	7
STIB 780	Kenya	Camel	1982	5
STIB 781	Kenya	Camel	1984	5
STIB 806K	China	Water buffalo	1983	7.5
Vietnam	Vietnam	Water buffalo	1998	7
***T. equiperdum* strains**				
Alfort	Unknown	Horse	1949	7
American	America	Horse	Unknown	6.5
AnTat 4.1	Unknown	Unknown	Unknown	5.5
ATCC30019	France	Horse	1903	5
ATCC30023	France	Horse	1903	5
BoTat 1.1	Morocco	Horse	1924	4
Canadian	Canada	Horse	Unknown	9
Hamburg	Unknown	Unknown	Unknown	7.5
OVI	South Africa	Horse	1977	5
STIB 818	China	Horse	1979	2
SVP	Unknown	Unknown	Unknown	5

-- denotes unsuccessful *in vivo* propagation

The drug sensitivities established for the nine *T. evansi* strains against the four current standard drugs are shown in *Table 2.2*. The STIB 806K *T. evansi* reference strain typified IC_{50} values considered to be sensitive to the drugs suramin (70.4), diminazene (4.5) and

cymelarsan (1.4). All other strains, except the two Kenyan strains and the Colombian strain, showed similar sensitivity to suramin. These two Kenyan strains isolated from camels (STIB 780 and STIB 781), produced IC_{50} values of 14'500 and 11'000 ng/ml respectively (around 200 times higher than the reference strain), demonstrating resistance to suramin, whilst a reduced sensitivity (four times greater than the reference strain) was observed in the Colombian strain with an IC_{50} value of 278.9 ng/ml.

Diminazene sensitivity is demonstrated in the reference strain, in the Kenyan strains (STIB 780 and STIB 781), the Kazakhstan strain and the South American strains (CAN86/Brazil and Colombia). A reduced sensitivity was seen in strains originating from the South East of Asia, in the Indonesian RoTat 1.2 and the Philippines strain, being approximately four times and five times, respectively, less sensitive than the reference strain. All *T. evansi* strains tested against cymelarsan produced IC_{50} values within the range of > 0.1 to 2.8 ng/ml. The Colombian strain showed the least sensitivity to quinapyramine (greater than six times that of the reference strain), followed by the CAN86/Brazil strain, the RoTat 1.2 strain, the Kazakhstan strain and the Philippines strain. The more sensitive *T. evansi* strains were STIB 780, STIB 781 and the Vietnam strain.

Also displayed in *Table 2.2* are the IC_{50} values for the eleven *T. equiperdum* strains tested. All strains demonstrated similar sensitivities for suramin as that of the reference strain, BoTat 1.1 (87.5 ng/ml), with the increasing exceptions of the strains, Hamburg, AnTat 4.1, ATCC30023 and American, which demonstrated approximately 1.5, 2, 3 and 4 -fold differences, respectively. The AnTat 4.1 strain revealed the highest sensitivity against diminazene, with five *T. equiperdum* strains showing similar diminazene sensitivity as those displayed by the majority of the *T. evansi* strains (Alfort, ATCC30023, BoTat 1.1 (reference strain), Hamburg and SVP strains). Both the American and the ATCC30019 strains revealed decreased sensitivity to diminazene, compared to the reference strain, by a factor of three, whilst the Canadian and STIB 818 strains revealed decreased sensitivities through a factor of four. The South African OVI strain was 70 times less sensitive to diminazene as the BoTat 1.1 reference strain, signifying resistance to this drug.

Similar to the *T. evansi* strains, all the *T. equiperdum* strains tested showed drug sensitivities of > 0.1 to 2.0 ng/ml against cymelarsan, apart from the OVI strain, which was twenty-five times higher than the reference value. The majority of *T. equiperdum* strains demonstrated quinapyramine sensitivities within the range of > 0.1 to 2.2 ng/ml. The reference strain (3.3 ng/ml) was thirteen times more sensitive than the Hamburg strain (43.8

ng/ml), fourteen times more sensitive than the Canadian strain (47.6 ng/ml) and twenty-three times more sensitive than the OVI strain (76.4 ng/ml) to quinapyramine.

Table 2.2. Mean drug sensitivities (given as IC_{50} in ng/ml) obtained for the four standard drugs tested against *Trypanosoma evansi* and *Trypanosoma equiperdum* study strains.

Strain	Standard drugs (inhibitory concentration (IC_{50}) values in ng/ml)							
	Suramin		Diminazene		Cymelarsan		Quinapyramine	
***T. evansi* strains**								
CAN86/Brazil	76.5	(±4.21)	2.7	(±0.28)	0.8	(±0.00)	15.8	(±0.35)
Colombia	278.9	(±5.84)	2.2	(±0.14)	0.5	(±0.07)	84.5	(±0.00)
Kazakhstan	97.8	(±1.48)	4.1	(±0.07)	1.1	(±0.14)	12.8	(±0.00)
Philippines	81.5	(±3.42)	20.2	(±0.35)	2.8	(±0.28)	7.4	(±4.24)
RoTat 1.2	69.5	(±6.99)	15.9	(±0.07)	2.2	(±0.00)	14.4	(±1.70)
STIB 780	14’500.0	(±0.00)	1.9	(±0.22)	0.2	(±0.07)	<0.1	(±0.00)
STIB 781	11’000.0	(±0.00)	5.4	(±0.42)	<0.1	(±0.00)	3.4	(±0.28)
STIB 806K	70.4	(±4.05)	4.5	(±0.07)	1.4	(±0.07)	13.3	(±0.57)
Vietnam	91.1	(±5.58)	8.2	(±0.71)	2.1	(±0.07)	3.0	(±0.28)
***T. equiperdum* strains**								
Alfort	76.9	(±5.91)	6.1	(±0.35)	1.3	(±0.09)	<0.1	(±0.00)
American	22.1	(±6.92)	13.5	(±0.07)	1.4	(±0.21)	<0.1	(±0.00)
AnTat 4.1	46.7	(±7.91)	<0.1	(±0.00)	<0.1	(±0.00)	2.2	(±2.83)
ATCC30019	31.4	(±2.84)	14.3	(±0.92)	0.9	(±0.07)	<0.1	(±0.00)
ATCC30023	75.6	(±4.17)	3.6	(±0.57)	1.8	(±0.28)	<0.1	(±0.00)
BoTat 1.1	87.5	(±3.30)	4.3	(±0.21)	0.7	(±0.00)	3.3	(±1.34)
Canadian	68.8	(±6.50)	19.0	(±0.57)	1.6	(±0.14)	47.6	(±0.57)
Hamburg	61.2	(±4.04)	5.5	(±0.42)	0.6	(±0.00)	43.8	(±1.49)
OVI	92.7	(±6.84)	302.5	(±12.1)	17.6	(±0.78)	76.4	(±2.47)
STIB 818	86.6	(±4.60)	19.2	(±0.00)	<0.1	(±0.00)	1.6	(±0.23)
SVP	71.1	(±10.6)	6.5	(±0.42)	2.0	(±0.00)	<0.1	(±0.00)

(±) denotes standard deviations of at least three to four experiments, each performed in duplicate.

Discussion

The aim of this study was to produce a drug profile for a panel of *Trypanosoma evansi* and *Trypanosoma equiperdum* strains, against the four standard drugs currently used for treatment of Surra (suramin, diminazene, cymelarsan and quinapyramine). With this collection, it was possible to demonstrate the presence of resistance and reduced drug sensitivity occurring in various locations around the world.

A major advantage of using the ^{3}H-hypoxanthine assay is that it allows original isolates, which have not yet been adapted to *in vitro* cultivation, to be tested under laboratory

conditions to determine sensitivities against certain drugs. The IC_{50} values produced in this way tend to reflect a more accurate sensitivity of a specific field isolate to a certain drug, than if the isolate had been culture adapted and kept under *in vitro* conditions for a long period of time, before it is then tested. However, in order to produce such drug sensitivity values, a high parasite starting concentration is required (in this study, $2x10^6$ / ml parasites were required for each isolate). Whilst many of the field isolates do not contain such high numbers of parasites, it is possible to pass them once through mice and hence establish higher parasitaemias, which in turn can be harvested and utilised in the ^{3}H-hypoxanthine assay. By means of an *in vivo* trypanosome propagation technique in mice, the majority of *T. evansi* and *T. equiperdum* study isolates were primarily propagated (parasitaemias were established within a week), then harvested, by passing the mouse blood through a DEAE-cellulose column, to finally achieve the high parasite concentrations needed to perform the drug sensitivity assays. Two *T. evansi* strains could not be successfully propagated in NMRI mice (Merzouga 56 and MHRYD/Brazil). Although parasites were detected after infection with these strains, no suitable trypanosome propagation could be carried out even after several mouse subpassages. These two strains were thus removed from the study and no further tests were performed with them.

The results produced by the *T. evansi* study strains (seen in *Table 2.2*) demonstrate that variations in drug sensitivities for the four standard drugs can occur between the geographically different strains. This is best seen in the two Kenyan strains isolated from camels (STIB 780 and STIB 781), both of which clearly display resistance to suramin with IC_{50} values of 14’500 and 11’000 ng/ml respectively. In terms of field application, ineffective treatment of these isolates with suramin suggests that either cymelarsan or quinapyramine could be used as an alternative. To add to this dilemma, not only are cymelarsan and quinapyramine not readily available in locations such as Africa, but they are also rather expensive. In addition, reduced suramin sensitivity was also shown in the Colombian strain (278.9 ng/ml), originally isolated from a horse in 1973.

The Indonesian RoTat 1.2 and the Philippines strains demonstrated the least diminazene sensitivity of all the *T. evansi* study strains. Since diminazene is used in these two Southeast Asian countries as the standard drug for treating *T. evansi* infections in cattle and buffalo and as resistance in the field tends to occur primarily through inadequate or erroneous application of subcurative doses of a drug over a period of time (*Bacchi, 1993*), the reduced sensitivities found in the RoTat 1.2 and the Philippines strains could be due to an

overexposure of diminazene treatment in water buffaloes and thus less sensitive strains against this drug.

A substantial reduction in diminazene sensitivity can be seen in the South African *T. equiperdum* OVI strain (302.5 ng/ml). Since *T. equiperdum* infections are not considered curable on a clinical basis and there are currently no officially approved drugs, it is uncertain as to whether the OVI strain has developed diminazene resistance through the misuse of chemotherapeutic regimens in the field. However, this high IC_{50} value seen with diminazene helps to explain the equally high IC_{50} value seen for cymelarsan (17.6 ng/ml). Previous reports have described a *TbAT1* gene found in trypanosomes, responsible for encoding a nucleoside P2 transporter (*Mäser et al, 1999*). This transporter is vital in the uptake of adenosine and adenine within trypanosomes, allowing them to salvage purines and nucleoside bases. Moreover, the P2 transporter is involved in the active drug uptake of diamidine compounds and melaminophenyl arsenicals. Furthermore, loss of this P2 transporter renders that organism resistant to such compounds (*Carter and Fairlamb, 1993*; *Carter et al., 1995*; *Barrett et al., 1995*). Therefore, if the OVI strain is missing a functional P2 transporter, it would be prevented from adequately taking up the drugs, diminazene and cymelarsan. Several studies have already shown the possible cross resistance between diamidine drugs, such as diminazene, and melaminophenyl arsenical compounds, such as cymelarsan (*Zhang et al., 1993*; *Ross and Barns, 1996*). Although cymelarsan is officially registered for chemotherapeutic use in horses, as well as in other animals, such as camels and goats, whether this drug is readily available on the veterinary market in South Africa is another issue. Due to the limited availability and high costs of cymelarsan, it is doubtful as to whether the OVI strain has lost part of its cymelarsan sensitivity through drug overexposure in the field, whereas cross resistance to another drug provides a more probable explanation.

The high IC_{50} value seen with quinapyramine for the South African OVI strain (76.4 ng/ml) could indicate additional cross resistance between quinapyramine, cymelarsan and diminazene. *Ndoutamia et al.* (*1993*) were able to induce quinapyramine resistance in *Trypanosoma congolense in vivo*, which was associated with cross resistance to isometamidium, homidium and diminazene. As cross resistance is frequently seen in drugs belonging to similar chemical families, this could be an explanation why the OVI strain demonstrates such reduced sensitivity against all three standard drugs (diminazene, cymelarsan and quinapyramine).

The use of quinapyramine as the drug of choice in Colombia to treat *T. evansi* infections in horses, could explain the reduced sensitivity to this drug. As the Colombian

strain was itself isolated from an equine host, the overuse and mismanagement of quinapyramine therapy in the field could be causing the emergence of potential resistance, which consequentially would lead to less sensitive *T. evansi* strains in South America. This could also be the case for the standard drug suramin, which was previously used to treat infected horses and cattle throughout South America, hence leading to the reduced sensitivity shown by the Colombian strain against suramin.

With this range of *T. evansi* and *T. equiperdum* isolates from around the world, a reference panel of isolates with known drug sensitivity could be established. The study has demonstrated drug resistance and reduced drug sensitivity in certain strains, a development which needs to be further monitored. Moreover, this leads to the suggestion that this drug profile could also be used as a reference when screening and testing new compounds against these two infectious agents. Since chemotherapy remains the most efficient and effective way to control Surra, it is vital that new, effective and preferably cheaper drugs are discovered to combat infection as soon as possible.

Acknowledgements

The authors wish to express gratitude towards ZR Lun (China), to the International Livestock Research Institute (Kenya) and to the Institute of Tropical Medicine (Belgium) for permitting the use of the various parasite strains in this study. This work was supported by COST B22 (Grant No. C04.0104), by the Swiss State Secretariat for Education and Research.

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In vitro activity and preliminary toxicity of various diamidine compounds against *Trypanosoma evansi*

Kirsten Gillingwater[1], David W Boykin[2], Richard R Tidwell[3] and Reto Brun[1]

[1]Parasite Chemotherapy, Swiss Tropical Institute, Socinstrasse 57, 4002 Basel, Switzerland

[2]Department of Chemistry, Georgia State University, Atlanta GA, United States of America

[3]Department of Pathology, University of North Carolina, Chapel Hill NC, United States of America

Abstract

Trypanosoma evansi is an animal pathogenic protozoan, causing a wasting disease called Surra. Transmitted mechanically by biting flies of the Genera, *Tabanus*, *Stomoxys* and *Lyperosia*, Surra is broadly distributed with a wide range of mammalian hosts. Chemotherapy is the most efficient control method with treatment for Surra currently dependent on four main drugs. Unfortunately, with the appearance of resistance to these drugs, their effective use is threatened, emphasising a need to find new drugs.

Diamidines (dicationic molecules) bind to the minor groove of DNA at AT-rich sites. They apply their biological activity by primarily binding to DNA and then inhibiting one or more DNA dependent enzymes or by directly impeding the transcription process. In total, 181 novel diamidine compounds were tested *in vitro* to determine activity against an animal pathogenic Chinese kinetoplastic *Trypanosoma evansi* strain. In comparison, a human pathogenic *Trypanosoma brucei rhodesiense* strain and a P2 transporter knock out of a *Trypanosoma brucei brucei* strain were also tested.

All diamidine compounds tested in this study against *T. evansi* produced inhibitory concentration (IC_{50}) values below 30 ng/ml; whereas 70 % of these compounds had IC_{50} values below 10 ng/ml. The results demonstrate that these compounds are highly active against *T. evansi in vitro*. In addition, preliminary *in vivo* toxicity tests were performed on all 181 novel diamidines with 55 % of the compounds showing no acute toxicity. In contrast, 8 % were toxic and 37 % were lethal within a mouse model.

Keywords: *Trypanosoma evansi*, Surra, diamidines, chemotherapy.

Introduction

Trypanosoma evansi is an animal pathogenic protozoan, which causes a wasting disease called Surra. Surra is widely distributed geographically and has a broad range of mammalian hosts, thus causing great economical loss in areas of Africa, Asia and South America (*Giardina et al., 2003*). Transmitted mechanically by biting flies (*Tabanus*, *Stomoxys* and *Lyperosia* species) (*Luckins, 1992*), *T. evansi* is pathogenic in most domestic animals and some wild animals, depending on the virulence of the strain and various host

factors (*Kaminsky et al., 1997*). The main host species varies according to geographical location (*Davila and Silva, 2000*; *Holland et al., 2004*; *Lun et al., 2004*; *Zhou et al., 2004*). The endemic geographic distribution of this disease ranges from China, the Philippines and parts of Indonesia, across Southeast Asia and parts of the former U.S.S.R, into the Indian subcontinent, the Middle East and Africa, until it reaches Central and South America (*OIE, 2004*).

Treatment for Surra is currently dependent on four drugs, namely suramin, diminazene aceturate, quinapyramine and cymelarsan (*Zhang et al., 1992*). While the first three have been utilised for more than 50 years (*Lourie and York, 1939*; *Kaminsky and Zweygarth, 1989*), cymelarsan, belonging to the family of melaminophenyl arsenicals, was pharmaceutically developed less than twenty years ago (*Zelleke et al., 1989*; *Raynaud et al., 1989*). With the rising appearance of resistance to these drugs, their effective use is threatened, emphasising the necessity for finding new alternative drugs (*Kaminsky et al., 1989*; *De Koning, 2001*; *Suswam et al., 2001*). Since many of the hosts affected in these endemic countries represent expensive and prestigious animals, a new drug against *T. evansi* infection would have a potentially high market value amongst other agents currently available for trypanosomal diseases.

An important step to consider in discovering any anti-parasitic drug is to determine the efficiency with which it can be taken up by the parasite. This can occur via one (or more) of four main processes; passive diffusion, endocytosis, receptor-mediated uptake or transporter-mediated uptake. Certain consequences, such as the mode of action, selectivity and potential development of resistance of a drug are derived and will in turn depend on the method of uptake used (*Carter et al., 1995*; *De Koning, 2001*). The selectivity of diamidines for example, is primarily due to the selective accumulation by the pathogen rather than by host cells (*Denise and Barrett, 2001*).

Diamidines are dicationic molecules, which bind to the minor groove of DNA at AT-rich sites. Their interaction with the DNA minor groove has been examined at a molecular level, concluding that these compounds apply their biological activity by primarily binding to DNA and then inhibiting one or more of the DNA dependent enzymes (such as topoisomerases or nucleases) or by directly impeding the transcription process (*Wilson et al., 2005*). The selective binding to kinetoplastic DNA has also been suggested to play a vital role in the action of aromatic diamidines against pathogens (*Francesconi et al., 1999*).

The aim of this study was to test 181 diamidine compounds, found to be active against *T. b. rhodesiense in vitro*, and determine their activity against *T. evansi* strains. Since *T.*

evansi is phylogenetically very close to *Trypanosoma brucei*, its drug sensitivity is very similar to that of the *T. brucei* trypanosomes. It was therefore hypothesised that diamidine compounds, found active against *T. brucei* subgroups, could also be effective against *T. evansi*. Selection of the 181 active diamidine compounds was hence based on the inhibitory concentration (IC_{50}) values and relative activity shown by a *T. b. rhodesiense* strain used in previous experimental procedures.

Materials and methods

Trypanosome stocks

Three different trypanosome strains were used in this study. The reference strain, *Trypanosoma evansi* (STIB 806K) is kinetoplastic and was isolated from a water buffalo in China in 1983. A human pathogenic *Trypanosoma brucei rhodesiense* (STIB 900) strain, isolated in 1982 from a male patient in Ifakara, Tanzania, was used as a comparison. The third strain used was a genetic knock out of a *T. b. brucei* strain (STIB 777S), which is missing the *TbAT1* gene encoding the nucleoside P2 transporter. All three strains were cloned and adapted to axenic culture conditions as described in *Baltz et al.* (*1985*).

In addition to these strains, six further *T. evansi* strains, isolated from various geographical regions, were tested against a selection of diamidines to determine strain sensitivity. These six strains (CAN86/Brazil, Colombia, Kazakhstan, Philippines, RoTat 1.2 (Indonesia) and Vietnam) were originally obtained from the cryobank of the Institute of Tropical Medicine (ITM) in Antwerp, Belgium, and then adapted to axenic culture conditions as described in *Baltz et al.* (*1985*).

Mammalian cell lines

Rat skeletal myoblast cells, designated L6 cells, were used for the *in vitro* cytotoxicity assay to evaluate the toxicity of the diamidine compounds tested. The L6 cell line cultures were kept in 25 ml tissue culture flasks (Becton Dickinson, USA) and were incubated at 37 °C and 5 % CO_2.

Mice

Female NMRI mice, weighing 22 - 25 g, were used for the *in vivo* preliminary toxicity tests. Mice were specific pathogen free (SPF) and were housed in standard Macrolon type II cages, at 22 °C and with a relative humidity of 60 - 70 %. The mice received pelleted food and water *ad libitum*.

Standard trypanocidal drugs

Suramin (Germanin® from Bayer, Leverkusen, Germany), diminazene aceturate (D-7770, Sigma, St Louis, MO, USA), cymelarsan (MelCy®, Rhône Mérieux, Toulouse, France) and quinapyramine sulphate (Trypacide®, May & Baker, Lagos, Nigeria) were used as the standard drugs in this study.

Diamidine compounds

The diamidine compounds were previously designed, synthesised and chosen by the chemists, David Boykin and Richard Tidwell, based on the compounds' chemical and physical properties, general biological stability and the overall costs involved in their manufacture. Previous knowledge on some structure-function relationships was also applied, to ensure the most active chemical groups and compositions were included within these newly designed compounds.

Culture medium

Bloodstream form trypanosomes were cultivated in Minimum Essential Medium (MEM) (powder, GIBCO/BRL, No: 11400-033) with Earle's salts, supplemented with 25 mM HEPES, 1 g/l additional glucose, 2.2 g/l $NaHCO_3$ and 10 ml/l MEM non-essential amino acids (50x concentration). The medium was then further supplemented by adding 1 % of a 2-mercaptoethanol stock (14 µl of 12 mM 2-mercaptoethanol was diluted in 10 ml of distilled water), 1 % of a stock consisting of 100 mM sodium pyruvate and 50 mM hypoxanthine and 15 % heat inactivated horse serum, according to *Baltz et al.* (*1985*). The complete medium is called Baltz MEM (BMEM).

The L6 (rat myoblast) cells were cultivated using RPMI 1640 medium (powder, Life Technologies) and supplemented with 25 mM HEPES and 2 g/l $NaHCO_3$. The medium was then further supplemented with 1.7 µM L-glutamine and 10 % heat inactivated foetal calf serum.

Stock solutions and dilutions

Stock solutions of 10 mg/ml of each compound were prepared and then stored frozen at -20 °C. From these stock solutions, further compound dilutions were made for use in the various *in vitro* cell viability assays, using the appropriate culture medium as a solvent. Compound dilutions were made fresh on the day of each experimental procedure.

In vitro cell viability assay

To determine the IC_{50} values for the compounds tested, the Alamar Blue® assay detailed in *Räz et al.* (*1997*) was used. In brief, 50 µl of BMEM were added to each well of a 96-well flat bottomed microtitre plate (Costar™, Corning, NY, USA), except for the final row. Instead, 75 µl of BMEM, containing two times the highest compound concentration, were placed into each well on the last row. Thereafter, serial compound dilutions were performed, whereby 25 µl from the final row were removed and mixed thoroughly with the contents of the row above. Again, 25 µl from this second-to-last row were then removed and mixed with the next row above and so on until all rows had achieved this series of 1:3 compound dilutions. The final 25 µl were discarded. However, no compound dilutions were made within the first row, since this row acted as a control. A further 50 µl of BMEM were then added to every third column along the 96-well plate, to provide background control wells containing no trypanosomes. Finally into all the remaining wells, 50 µl of a trypanosome suspension were added. The trypanosome density was calculated using a cell counter and analyser system (CASY, Schaerfe System, Reutlingen, Germany) to enable a trypanosome seeding density of $4x10^4$/ml of culture medium to be added to the assay. The assay plates were then incubated at 37 °C with 5 % CO_2 for 69 hours, whereupon the plates were removed from the incubator and 10 µl of Resazurin dye (12.5 mg in 100 ml phosphate buffered saline) (Aldrich/Fluka, #33934, Buchs, Switzerland) were placed into each well. The assay plates were returned once again to the incubator for a further 3 hours under the same incubation conditions. Once the assay plates were ready, they were removed and read using a fluorescent

reader (SpectraMax, Gemini XS, Bucher Biotec, Basel, Switzerland) at an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC_{50} values were determined using appropriate computer software (SOFTmax Pro 3.1.2). All experiments were performed on all trypanosome strains in two separate experimental procedures.

In vitro cell cytotoxicity assay

To determine the cytotoxicity of each of the compounds tested, a standard operating procedure (SOP) using the L6 (rat skeletal myoblast) cell line was followed. It is similar to that of the Alamar Blue® assay, except for a few modifications. First, 100 µl of a $4x10^4$cells/ml cell suspension were added to each well of a 96-well flat bottomed microtitre plate (Costar™, Corning, NY, USA), except for the wells in every third column. The cell suspension was calculated using a Neubauer counting chamber and then diluted with culture medium to obtain the correct concentration. Into all other remaining wells, 100 µl of culture medium were placed without cells, acting as control wells. The plates were then incubated at 37 °C and 5 % CO_2 overnight, to enable the L6 cells to attach to the microtitre plates. The following day, the medium was removed from all the wells and all rows (except the last row) replaced with 100 µl of fresh culture medium. Thereafter, 150 µl of culture medium containing two times the highest compound concentration were added to the wells in the last row. Serial compound dilutions were performed by removing 50 µl from the last row and mixing them thoroughly with the contents of the wells in the second-to-last row. Then a further 50 µl from this row were removed and mixed thoroughly with the row above it and so on until all rows had achieved this compound dilution factor of 1:3. The final 50 µl were discarded. Again the top row of the microtitre plate was not included in the serial compound dilutions, since this row represented a control. The assay plates were then placed into the incubator at 37 °C with 5 % CO_2 for 69 hours. After this time had elapsed, the plates were removed from the incubator and 10 µl of Resazurin dye (Aldrich/Fluka, #33934, Buchs, Switzerland) were placed into each well. The assay plates were returned once again to the incubator for a further 3 hours under the same incubation conditions. Once the assay plates were ready, they were removed and read using a fluorescent reader (SpectraMax, Gemini XS, Bucher Biotec, Basel, Switzerland) at an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The IC_{50} values were determined using appropriate computer software (SOFTmax Pro 3.1.2). All experiments were performed in three separate experimental procedures.

In vivo preliminary toxicity tests

For the *in vivo* preliminary toxicity tests, an SOP was followed. The compounds were diluted freshly on the day of testing and injected into the mice using an intra-peritoneal (i.p.) route. A female NMRI mouse was taken and given an i.p. injection at 5 mg/kg. The mouse was then injected every 2 hours with an increasing dose, starting with the 5 mg/kg dose, then rising to 15 mg/kg, 30 mg/kg and 50 mg/kg. The cumulative dose corresponds to the injection dose, so that 15 mg/kg has an accumulation of 20, 30 mg/kg of 50 and 50 mg/kg of 100, respectively. During each 2 hour interval, the mouse was observed carefully for any signs of toxicity or sub-toxicity. After the final dose (50 mg/kg) had been administered, the mouse was observed for a further 2 hours and then again after 24 hours. The results of the *in vivo* preliminary toxicity tests were based on each compound being lethal (hence the mouse died), toxic (where the mouse showed signs of toxicity, but did not die) or not toxic (where the mouse appeared normal and could tolerate the highest accumulated dose of 100 mg/kg).

Results

In total, the four standard drugs against *Trypanosoma evansi* and 181 novel diamidine compounds were tested *in vitro* using a cell viability assay. For each compound, inhibitory concentration (IC_{50}) values in ng/ml were obtained, against three different trypanosome strains; an animal pathogenic Chinese kinetoplastic *T. evansi* (STIB 806K), a human pathogenic *T. b. rhodesiense* (STIB 900) and a P2 transporter knock out of a *T. b. brucei* (TbAT1, originally STIB 777S). The IC_{50} values for the four standard drugs are shown in parallel in *Table 3.1* for each of the three trypanosome strains, respectively.

The greater IC_{50} values seen for suramin (a naphthalene compound) clearly demonstrate that this current standard drug has reduced activity *in vitro* against both STIB 806K and STIB 900. Its value for the TbAT1 knock out strain resembles a drug that still has inadequate trypanocidal activity, even though it relies mainly on diffusion and/or endocytosis for its uptake. Diminazene (a dicationic diamidine) has a low IC_{50} value against STIB 900, yet gives a slightly elevated value for STIB 806K. Although it seems to be reasonably active against *T. evansi*, its value for the TbAT1 strain shows that it relies heavily on P2 transport, which in turn could cause problems with resistant strains. *In vitro* IC_{50} values for cymelarsan

(a member of the melaminophenyl arsenicals) are much lower than the two previous standard drugs against STIB 806K and STIB 900. The fourth standard drug, quinapyramine (belonging to the quinoline pyrimidines), reveals even lower IC_{50} values for STIB 806K and STIB 900 than the other standard drugs. However, an elevated IC_{50} value for the TbAT1 strain demonstrates an involvement with P2 transport.

Table 3.1. The four standard drugs currently used against *T. evansi* infection, with their chemical families and structures depicted alongside the IC_{50} values obtained with a kinetoplastic *T. evansi* strain (STIB 806K), a *T. b. rhodesiense* strain (STIB 900) and a *T. b. brucei* P2 transporter knock out strain (TbAT1). Cytotoxicity IC_{50} values (using L6 cells) and preliminary *in vivo* toxicity values (using NMRI mice) are also shown.

Drug identity	**Chemical family**	**Inhibitory concentration (IC_{50}) in ng/ml**				**Preliminary toxicity (mg/kg)**	**Chemical structure**
		STIB 806K	STIB 900	TbAT1 (K.O.)	Cytotoxicity (L6 cells)		
Suramin	Naphtha-lene	87.6	75.8	>100	>90’000	Toxic at 50 i.p.	
Diminazene	Triazene	12.5	2.8	>100	4’091	Lethal at 20 i.p.	
Cymelarsan	Melamino-phenyl arsenical	1.1	1.9	3.3	>90’000	Lethal at 20 i.p.	
Quina-pyramine	Quinoline pyrimidine	0.1	0.3	>87.4	83’623	Lethal at 5 i.p.	

i.p. denotes an intra-peritoneal route was used

The IC_{50} values for the Boykin compounds against all three trypanosome strains can be seen in *Table 3.2*. All compounds produced an IC_{50} value below 20 ng/ml for both the STIB 806K and STIB 900 strains. In addition, eighty-eight of these compounds produced an IC_{50} value below 10 ng/ml, again for both STIB 806K and STIB 900 strains. These compounds demonstrate higher *in vitro* activity against the *T. evansi* strain than the standard drugs provide. Therefore, compounds showing IC_{50} values of less than 10 ng/ml can be selected for further investigation. The IC_{50} values for the TbAT1 knock out strain for the Boykin compounds ranges from 0.1 ng/ml to greater than 93.7 ng/ml. The compounds showing higher IC_{50} results against this knock out strain can be selected against, since these compounds indicate probable P2 transport and hence have less potential activity against drug resistant *T. evansi* strains. In summary, only those compounds demonstrating high activity against the STIB 806K and the TbAT1 strains will be selected for based on the current selective criteria. This implies all compounds with low IC_{50} values against both STIB 806K and TbAT1 strains will continue on into *in vivo* investigations.

Table 3.2. Ninety-eight new diamidine compounds, designed by David Boykin, with their chemical families and structures depicted alongside the IC_{50} values obtained with a kinetoplastic *T. evansi* strain (STIB 806K), a *T. b. rhodesiense* strain (STIB 900) and a *T. b. brucei* P2 transporter knock out strain (TbAT1). Cytotoxicity IC_{50} values (using L6 cells) and preliminary *in vivo* toxicity values (using NMRI mice) are also shown.

Compound identity	Chemical family	Inhibitory concentration (IC_{50}) in ng/ml				Preliminary toxicity (mg/kg)	Chemical structure
		STIB 806K	STIB 900	TbAT1 (K.O.)	Cytotoxicity (L6 cells)		
DB 75	Diphenyl furan	2.3	0.8	15.4	>90’000	Toxic at 50 i.p.	
DB 103	Diphenyl furan	6.0	9.1	18.5	>90’000	Lethal at 50 i.p.	
DB 201	Benz-imidazole	4.5	8.9	20.9	>90’000	Toxic at 20 i.p.	
DB 210	Benz-imidazole	1.2	9.6	18.3	>90’000	Lethal at 100 i.p.	
DB 211	Pyrimidine	10.4	3.9	4.7	>90’000	Not toxic at 100 i.p.	
DB 217	Benz-imidazole	2.2	3.2	3.9	>90’000	Lethal at 50 i.p.	
DB 242	Diphenyl furan	9.8	8.6	7.9	>90’000	Lethal at 100 i.p.	
DB 262	Diphenyl pyrrole	8.2	4.6	4.4	85’280	Not toxic at 100 i.p.	
DB 320	Diphenyl pyrrole	5.0	2.1	2.8	>90’000	Not toxic at 100 i.p.	
DB 325	Benz-imidazole	2.8	4.2	3.1	>90’000	Not toxic at 100 i.p.	
DB 346	Biphenyl	5.8	2.4	1.2	6’500	Not toxic at 100 i.p.	
DB 351	Diphenyl thiophene	3.3	0.7	0.8	88’410	Not toxic at 100 i.p.	
DB 417	Diphenyl furan	3.6	2.9	3.6	>90’000	Lethal at 100 i.p.	
DB 427	Diphenyl furan	7.2	4.1	5.7	89’530	Toxic at 50 i.p.	
DB 484	Diphenyl oxazole	6.5	1.5	3.2	>90’000	Lethal at 100 i.p.	
DB 497	Benz-imidazole	8.5	12.2	6.0	85’220	Toxic at 20 i.p.	

Table 3.2. **continued…**

DB 508	Benz-imidazole	9.6	2.4	5.1	>90'000	Toxic at 20 i.p.
DB 544	Diphenyl furan	3.7	3.4	6.0	>90'000	Toxic at 100 i.p.
DB 545	Diphenyl Furan	8.6	3.8	12.5	6'860	Toxic at 20 i.p.
DB 560	Diphenyl thiophene	3.7	1.8	2.6	>90'000	Toxic at 20 i.p.
DB 609	Diaryl furan	8.3	6.2	18.5	>90'000	Toxic at 20 i.p.
DB 690	Diphenyl furan	7.3	1.8	2.0	>90'000	Not toxic at 100 i.p.
DB 746	Diphenyl furan	10.6	9.0	13.5	53'490	Toxic at 20 i.p.
DB 763	Diphenyl furan	2.7	6.6	5.7	>90'000	Toxic at 50 i.p.
DB 773	Thiophene	3.7	4.5	71.9	>90'000	Lethal at 100 i.p.
DB 820	Aza-furan	5.4	1.3	3.0	>90'000	Not toxic at 100 i.p.
DB 828	Diaryl furan	5.3	5.7	75.6	>90'000	Not toxic at 100 i.p.
DB 832	Bifuran	8.7	3.6	6.1	>90'000	Not toxic at 100 i.p.
DB 841	Indene	7.2	8.7	82.0	>90'000	Lethal at 100 i.p.
DB 846	Benz-imidazole	10.0	10.7	5.0	>90'000	Lethal at 50 i.p.
DB 849	Indenone	1.8	3.7	6.4	>90'000	Toxic at 50 i.p.
DB 850	Benz-imidazole	4.5	5.3	9.2	>90'000	Not toxic at 100 i.p.
DB 851	Benz-imidazole	7.4	12.9	15.5	>90'000	Not toxic at 100 i.p.
DB 853	Benz-imidazole	7.2	13.7	24.4	>90'000	Not toxic at 100 i.p.
DB 866	Thiophene	3.4	2.4	25.6	>90'000	Lethal at 100 i.p.
DB 867	Aza-furan	1.7	0.8	1.1	>90'000	Not toxic at 100 i.p.

***Table 3.2.* continued…**

DB 877	Biphenyl	3.4	0.2	0.2	>90’000	Lethal at 100 i.p.
DB 902	Diphenyl furan	3.5	7.4	28.9	>90’000	Lethal at 100 i.p.
DB 911	Benz-imidazole	3.7	2.4	2.4	>90’000	Lethal at 100 i.p.
DB 930	Diphenyl furan	4.3	1.8	19.5	>90’000	Not toxic at 100 i.p.
DB 935	Aza-furan	4.6	7.3	84.0	>90’000	Lethal at 100 i.p.
DB 943	Biphenyl	2.8	4.5	3.9	88’070	Not toxic at 100 i.p.
DB 945	Diphenyl furan	3.2	2.9	3.8	>90’000	Not toxic at 100 i.p.
DB 988	Benz-imidazole	5.6	3.5	54.5	>90’000	Not toxic at 100 i.p.
DB 989	Benz-imidazole	7.1	4.5	3.6	>90’000	Not toxic at 100 i.p.
DB 994	Aza-furan	6.6	1.1	1.2	37’850	Not toxic at 100 i.p.
DB 1012	Diaryl furan	7.2	3.3	2.7	>90’000	Not toxic at 100 i.p.
DB 1016	Triphenyl furan	10.5	6.9	9.2	>90’000	Lethal at 100 i.p.
DB 1017	Diphenyl furan	8.0	2.9	4.9	>90’000	Not toxic at 100 i.p.
DB 1019	Biphenyl	2.8	0.4	0.5	>90’000	Not toxic at 100 i.p.
DB 1023	Diphenyl thiophene	6.5	4.3	6.6	>90’000	Lethal at 100 i.p.
DB 1044	Diaryl pyridine	11.6	6.7	5.3	>90’000	Not toxic at 100 i.p.
DB 1046	Benz-imidazole	3.2	4.2	>93.7	>90’000	Not toxic at 100 i.p.
DB 1049	Aza-triaryl	7.0	9.2	15.7	>90’000	Lethal at 100 i.p.
DB 1050	Aza-triaryl	6.3	1.8	8.1	>90’000	Not toxic at 100 i.p.
DB 1052	Thiazole	10.2	7.2	7.1	41’570	Not toxic at 100 i.p.

***Table 3.2.* continued…**

DB 1055	Benz-imidazole	11.4	11.8	3.4	>90'000	Not toxic at 100 i.p.	
DB 1065	Pyrrole	2.4	0.8	0.1	>90'000	Lethal at 100 i.p.	
DB 1077	Thiophene	8.2	3.4	61.2	>90'000	Not toxic at 100 i.p.	
DB 1114	Biphenyl	1.7	4.1	5.7	>90'000	Lethal at 100 i.p.	
DB 1149	Diaryl thiophene	6.1	4.3	4.3	>90'000	Not toxic at 100 i.p.	
DB 1152	Cinnamide	3.7	0.5	0.7	33'490	Lethal at 100 i.p.	Structure not shown due to patent reasons
DB 1157	Terphenyl	3.5	12.6	11.8	88'840	Not toxic at 100 i.p.	
DB 1159	Benz-oxazole	3.8	10.2	5.9	>90'000	Lethal at 100 i.p.	
DB 1164	Aza-terphenyl	8.9	0.3	0.2	>90'000	Lethal at 100 i.p.	
DB 1165	Terphenyl	2.4	1.3	1.2	>90'000	Lethal at 100 i.p.	
DB 1171	Indole	1.3	0.8	0.7	62'830	Not toxic at 100 i.p.	
DB 1172	Indole	3.3	4.6	2.9	>90'000	Not toxic at 100 i.p.	
DB 1173	Diaryl furan	6.2	11.4	11.3	>90'000	Not toxic at 100 i.p.	
DB 1177	Biphenyl	5.0	2.7	3.7	>90'000	Not toxic at 100 i.p.	
DB 1191	Indole	3.8	8.3	9.5	>90'000	Not toxic at 100 i.p.	
DB 1192	Indole	10.5	0.4	0.5	>90'000	Not toxic at 100 i.p.	
DB 1194	Diaryl thiophene	1.4	2.1	1.6	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
DB 1197	Benz-imidazole	2.9	9.9	15.0	>90'000	Lethal at 100 i.p.	
DB 1206	Terphenyl	8.7	0.9	0.2	>90'000	Not toxic at 100 i.p.	
DB 1210	Pyrazine	1.3	1.6	3.2	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons

***Table 3.2.* continued…**

DB 1213	Diaryl seleno-phene	10.0	0.9	0.6	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
DB 1214	Diaryl thiophene	1.3	5.9	4.4	>90'000	Lethal at 100 i.p.	
DB 1220	Terphenyl	5.9	12.2	16.9	>90'000	Not toxic at 100 i.p.	
DB 1228	Aza-terphenyl	11.5	2.9	3.0	>90'000	Lethal at 100 i.p.	
DB 1236	Benz-imidazole	9.5	4.8	5.2	>90'000	Not toxic at 100 i.p.	
DB 1237	Aza-terphenyl	6.9	7.9	9.3	>90'000	Lethal at 100 i.p.	
DB 1239	Aza-naphtha-lene	13.2	3.2	4.7	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
DB 1242	Aza-triaryl	3.7	5.7	5.7	>90'000	Not toxic at 100 i.p.	
DB 1250	Benz-imidazole	6.4	1.2	1.3	>90'000	Not toxic at 100 i.p.	
DB 1253	Aza-triaryl	3.2	5.7	4.3	>90'000	Not toxic at 100 i.p.	
DB 1255	Bithio-phene	5.6	7.8	6.0	>90'000	Not toxic at 100 i.p.	
DB 1258	Aza-terphenyl	8.3	9.5	7.5	>90'000	Not toxic at 100 i.p.	
DB 1265	Indene	8.2	4.1	4.0	>90'000	Not toxic at 100 i.p.	
DB 1266	Arylamide	8.0	7.7	9.8	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
DB 1271	Arylamide	7.0	8.1	9.8	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
DB 1272	Seleno-phene	5.7	5.1	6.8	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
DB 1283	Di-aza furamidine	6.5	2.4	2.7	>90'000	Not toxic at 100 i.p.	
DB 1288	Diaryl seleno-phene	5.4	0.9	0.7	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
DB 1340	Seleno-phene	3.8	0.9	1.2	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
DB 1342	Pyrrole	3.8	6.0	7.7	>90'000	Not toxic at 100 i.p.	

Table 3.2. **continued…**

DB 1370	Aza-pyridine	5.1	2.0	0.5	>90'000	Not toxic at 100 i.p.	
DB 1371	Aza-pyridine	10.8	16.0	12.0	>90'000	Not toxic at 100 i.p.	

i.p. denotes an intra-peritoneal route was used

In comparison, the IC_{50} values for the Tidwell compounds, tested against all three trypanosome strains, can be seen in *Table 3.3*. All the compounds produced IC_{50} values below 30 ng/ml for the *T. evansi* and *T. b. rhodesiense* strains, yet forty-one of these compounds demonstrated higher activity for the *T. evansi* (STIB 806K) strain, with IC_{50} results below 10 ng/ml. Again, compounds showing greater efficacy *in vitro* than the standard drugs are positively selected for continued investigation for an alternative compound against *T. evansi* infection. The IC_{50} values for the TbAT1 knock out strain for the Tidwell compounds ranged from 0.7 ng/ml to greater than 100 ng/ml. Since an important selective criterion is to avoid potential resistance occurring against new alternative compounds for *T. evansi* infection, only compounds with low IC_{50} values against the TbAT1 knock out strain will be pursued further.

Table 3.3. Eighty-three new diamidine compounds, designed by Richard Tidwell, with their chemical families and structures depicted alongside the IC_{50} values obtained with a kinetoplastic *T. evansi* strain (STIB 806K), a *T. b. rhodesiense* strain (STIB 900) and a *T. b. brucei* P2 transporter knock out strain (TbAT1). Cytotoxicity IC_{50} values (using L6 cells) and preliminary *in vivo* toxicity values (using NMRI mice) are also shown.

Compound identity	**Chemical family**	**Inhibitory concentration (IC_{50}) in ng/ml**				**Preliminary toxicity (mg/kg)**	**Chemical structure**
		STIB 806K	STIB 900	TbAT1 (K.O.)	Cytotoxicity (L6 cells)		
3 HXC 023	Benz-imidazole	9.5	13.0	4.2	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
5 BGR 066	Benz-imidazole	14.6	17.2	5.7	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
5 BGR 068	Benz-imidazole	7.5	14.4	6.9	>90'000	Lethal at 20 i.p.	Structure not shown due to patent reasons
5 BGR 086	Benz-imidazole	5.6	5.5	2.4	>90'000	Lethal at 20 i.p.	Structure not shown due to patent reasons
5 BGR 088	Benz-imidazole	10.3	8.8	5.5	>90'000	Lethal at 20 i.p.	Structure not shown due to patent reasons
5 BGR 094	Benz-imidazole	24.7	16.1	7.9	>90'000	Lethal at 20 i.p.	Structure not shown due to patent reasons

***Table 3.3.* continued…**

5 BGR 096	Benz-imidazole	27.7	23.6	8.3	>90'000	Lethal at 20 i.p.	Structure not shown due to patent reasons
6 BGR 012	Benz-imidazole	24.5	26.0	3.7	>90'000	Lethal at 20 i.p.	Structure not shown due to patent reasons
1 SMB 015	Xylene	9.8	7.4	97.9	>90'000	Toxic at 100 i.p.	
6 EVK 012	Isoxazole	21.4	26.2	20.6	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
1 RRT 039	Xylene	2.9	0.9	30.7	>90'000	Lethal at 50 i.p.	
6 MAA 143	Xylene	28.1	21.1	>100	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
5 MAA 085	Pentamidine analogue	22.6	26.2	>100	>90'000	Not toxic at 100 i.p.	
1 KAO 045	Pentamidine analogue	14.9	16.9	55.8	>90'000	Lethal at 100 i.p.	
3 STL 057	Pentamidine analogue	4.4	4.4	18.7	>90'000	Not toxic at 100 i.p.	
5 MAA 083	Pentamidine analogue	26.1	21.4	>100	>90'000	Not toxic at 100 i.p.	
5 MAA 101	Pentamidine analogue	11.2	9.8	>92.8	>90'000	Not toxic at 100 i.p.	
5 MAA 121	Pentamidine analogue	19.9	15.7	>100	>90'000	Not toxic at 100 i.p.	
5 MAA 123	Pentamidine analogue	24.5	24.2	98.2	>90'000	Lethal at 100 i.p.	
5 MAA 137	Pentamidine analogue	26.1	22.2	86.2	>90'000	Lethal at 50 i.p.	Structure not shown due to patent reasons
6 MAA 025	Pentamidine analogue	2.4	3.1	27.3	>90'000	Not toxic at 100 i.p.	
2 SAB 087	Benzo-furan	25.8	22.3	85.4	>90'000	Lethal at 50 i.p.	Structure not shown due to patent reasons
3 SMB 051	Bis-Benzo-furan	9.2	6.6	>100	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
3 SMB 065	Bis-Benzo-furan	7.5	9.9	93.6	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
3 SMB 079	Pentamidine analogue	20.9	26.4	>100	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
3 SMB 101	Pentamidine analogue	6.4	4.2	63.8	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons

***Table 3.3.* continued…**

4 SMB 058	Bis-Benzo-furan	24.1	24.5	>100	>90'000	Lethal at 100 i.p.	
4 SMB 092	Bis-Benzo-furan	18.9	13.2	>100	>90'000	Not toxic at 100 i.p.	
5 SMB 032	Bis-Benzo-furan	4.4	2.8	90.2	>90'000	Not toxic at 100 i.p.	
1 EVK 057	Pentamidine analogue	6.0	6.2	34.4	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
16 DAP 022	Carbazole	24.9	25.9	>100	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
4 SAB 075	Isoxazole	2.6	3.5	28.5	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
5 SMB 093	Isoxazole	22.2	11.5	17.3	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
16 DAP 095	Isoxazole	15.5	5.8	12.7	51'150	Not toxic at 100 i.p.	
3 KEG 083	Pentamidine analogue	8.2	11.5	36.3	>90'000	Not toxic at 100 i.p.	
3 NAN 027	Pentamidine analogue	9.0	2.5	43.1	>90'000	Lethal at 50 i.p.	Structure not shown due to patent reasons
6 SAB 038	Benzo-furan	11.8	11.4	6.1	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
8 SMB 021	Isoxazole	4.2	4.8	0.9	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
7 SAB 004	Benzo-furan	3.6	4.9	2.7	22'360	Not toxic at 100 i.p.	
7 SAB 015	Benzo-furan	10.3	8.1	5.9	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
7 EVK 097	Isoxazole	2.1	2.1	13.8	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
7 SAB 079	Isoxazole	3.1	2.4	1.9	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
8 EVK 024	Isoxazole	2.7	2.8	42.3	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
8 EVK 030	Isoxazole	2.1	4.8	58.7	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
19 DAP 021	Isoxazole	10.9	6.1	3.9	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
19 DAP 025	Naphthylene	1.8	1.5	1.8	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons

***Table 3.3.* continued…**

1 FMS 034	Phenoxy amidine	9.6	5.5	8.6	>90'000	Not toxic at 100 i.p.	
1 MCC 112	Phenoxy amidine	2.4	2.3	2.7	>90'000	Not toxic at 100 i.p.	
1 MCC 179	Phenoxy amidine	2.9	0.4	0.7	>90'000	Not toxic at 100 i.p.	
1 MCC 097	Phenoxy amidine	14.5	9.0	26.6	>90'000	Not toxic at 100 i.p.	
1 MCC 128	Phenoxy amidine	13.3	2.7	2.9	>90'000	Lethal at 100 i.p.	
1 MCC 148	Phenoxy amidine	3.3	1.1	1.8	>90'000	Not toxic at 100 i.p.	
1 MCC 184	Phenoxy amidine	4.3	4.8	4.3	>90'000	Not toxic at 100 i.p.	
10 SMB 038	Isoxazole	18.7	10.1	11.2	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
0 DWB 082	Biphenyl	12.5	3.5	3.1	24'740	Not toxic at 100 i.p.	
0 MXB 736	Phenoxy amidine	25.3	28.2	24.3	>90'000	Not toxic at 100 i.p.	
0 MXB 767	Phenoxy amidine	13.1	20.6	16.1	33'810	Not toxic at 100 i.p.	
0 MXB 864	Phenoxy amidine	7.0	9.2	9.2	>90'000	Not toxic at 100 i.p.	
10 SMB 084	Isoxazole	35.9	31.9	47.6	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
11 SMB 003	Pentamidine analogue	13.4	10.5	32.3	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
19 DAP 075	Isoxazole	15.0	12.7	12.1	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
19 DAP 085	Naphthylene	22.0	16.7	24.0	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
19 DAP 089	Isoxazole	6.7	10.7	13.7	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
20 DAP 001	Isoxazole	7.6	8.4	9.6	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
19 DAP 079	Isoxazole	17.6	12.3	10.7	>90'000	Lethal at 100 i.p.	Structure not shown due to patent reasons
8 SAB 066	Isoxazole	3.9	1.3	1.1	>90'000	Not toxic at 100 i.p.	Structure not shown due to patent reasons

***Table 3.3.* continued…**

8 SAB 068	Isoxazole	3.7	0.9	0.9	>90’000	Lethal at 100 i.p.	Structure not shown due to patent reasons
8 SAB 072	Isoxazole	8.0	4.6	4.5	>90’000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
20 DAP 055	Isoxazole	7.9	6.1	6.6	>90’000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
20 DAP 059	Isoxazole	13.8	4.2	4.0	>90’000	Lethal at 100 i.p.	Structure not shown due to patent reasons
12 SMB 003	Isoxazole	5.4	2.6	2.6	>90’000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
12 SMB 028	Furan	2.5	8.9	7.0	>90’000	Lethal at 100 i.p.	Structure not shown due to patent reasons
21 DAP 023	Pentamidine analogue	5.0	2.0	2.0	>90’000	Lethal at 100 i.p.	Structure not shown due to patent reasons
9 SAB 021	Isoxazole	13.3	11.6	11.0	>90’000	Not toxic at 100 i.p.	Structure not shown due to patent reasons
12 SMB 011	Isoxazole	28.5	26.0	97.0	>90’000	Lethal at 100 i.p.	Structure not shown due to patent reasons
21 DAP 049	Isoxazole	7.4	5.4	9.8	>90’000	Lethal at 100 i.p.	Structure not shown due to patent reasons
9 SAB 072	Phenoxy amidine	11.3	10.1	12.1	>90’000	Toxic at 100 i.p.	
9 SAB 076	Phenoxy amidine	7.6	3.1	4.3	>90’000	Lethal at 100 i.p.	
9 SAB 078	Phenoxy amidine	7.1	3.5	7.1	>90’000	Lethal at 100 i.p.	
8 SAB 070	Isoxazole	13.5	9.4	19.9	>90’000	Lethal at 100 i.p.	Structure not shown due to patent reasons
21 DAP 046	Isoxazole	14.1	6.1	7.1	>90’000	Lethal at 100 i.p.	Structure not shown due to patent reasons
10 SAB 037	Triazole	5.0	4.5	4.9	>90’000	Lethal at 100 i.p.	Structure not shown due to patent reasons
10 SAB 055	Triazole	4.8	2.4	2.9	>90’000	Lethal at 100 i.p.	Structure not shown due to patent reasons

i.p. denotes an intra-peritoneal route was used

Each of the 181 compounds, including the four standard drugs, was applied to an *in vitro* cell cytotoxicity assay using L6 cells. Their IC_{50} values in ng/ml are depicted in *Table 3.1* for the standard drugs, *Table 3.2* for the Boykin compounds and *Table 3.3* for the Tidwell compounds. Compounds which were considered non-cytotoxic to the L6 (rat myoblast) cells

had an IC_{50} value greater than 90'000 ng/ml (>90'000). The important exceptions to this were the standard drug, diminazene, the following Boykin compounds, DB 346, DB 545, DB 746, DB 994, DB 1052, DB 1152 and DB 1171 and the following Tidwell compounds, 16 DAP 095, 7 SAB 004, 0 DWB 082 and 0 MXB 767.

To evaluate the toxicity of these compounds within an *in vivo* system, preliminary *in vivo* toxicity tests were performed using an NMRI mouse model. Each compound was administered at an increasing accumulative dose using an intra-peritoneal application route in mg/kg. The mice were observed for signs of acute toxicity within a 48 hour period and then grouped accordingly. Compounds which caused death to the mouse were classed as lethal, whereas compounds showing no acute toxicity were defined as not toxic, up to a maximum accumulative dose of 100 mg/kg. Compounds that demonstrated some form of acute toxicity, but not enough to cause death, were labelled as toxic at that given accumulative dose, also up to a maximum accumulative dose of 100 mg/kg.

In *Table 3.1*, the preliminary *in vivo* toxicity results show that three of the standard drugs (diminazene, cymelarsan and quinapyramine) are actually lethal when doses of 20 mg/kg, 20 mg/kg and 5 mg/kg are given intra-peritoneally, respectively. The remaining standard drug, suramin, is not lethal, but toxic after an accumulative dose of just 50 mg/kg. The *in vivo* preliminary toxicity results for the Boykin compounds can be seen in more detail in *Table 3.2*. In total, twenty-eight of these compounds were lethal at 100 mg/kg, three were lethal at 50 mg/kg, only one was toxic at 100 mg/kg, four compounds were toxic at 50 mg/kg and seven were toxic at 20 mg/kg accumulative dose. Similarly, the *in vivo* preliminary toxicity results for the Tidwell compounds are shown in *Table 3.3*. In total, twenty-seven of these compounds were lethal at 100 mg/kg, four were lethal at 50 mg/kg, six were lethal at just 20 mg/kg and two compounds were toxic at 100 mg/kg accumulative dose.

The toxicity of a compound *in vivo* remains another important criterion for selecting alternative compounds as potential leads against *T. evansi* infection. A compound which has the ability to treat infected animals without causing harm to the organism itself, would provide many ideal properties, hence compounds which demonstrate no preliminary toxicity in the mouse model used, are selected for further analysis within *in vivo* models. In total, fifty-five compounds tested from David Boykin and forty-four compounds tested from Richard Tidwell were found to be not toxic, after the highest accumulated dose of 100 mg/kg was examined, in a mouse model.

A compound capable of treating several strains of *T. evansi* would be an additional ideal property for an alternative novel drug against infection. Since high activity was seen by

these dicationic compounds against the reference Chinese *T. evansi* strain (STIB 806K), a select few were thus chosen to be tested against seven different *T. evansi* strains, obtained from geographically separate locations. The standard drugs were included to determine how sensitive each strain was to currently available drugs, whilst the six selected diamidine compounds were chosen based on their curative efficacy in a preliminary mouse model, the data of which can be viewed in the next chapter.

The IC_{50} values (in ng/ml) obtained for the *in vitro* strain sensitivity study can be viewed in *Table 3.4*. The STIB 806K strain (marked in blue) represents the reference strain used for this study. All *T. evansi* strains tested (including the reference strain) showed similar sensitivities for diminazene, cymelarsan and quinapyramine. For suramin however, the IC_{50} values fluctuated from 44.0 ng/ml to 87.6 ng/ml.

Table 3.4. *In vitro* strain sensitivities (shown as IC_{50} values in ng/ml) of seven different *T. evansi* strains, originating from geographically separate locations, against the four standard drugs currently in use against *T. evansi* infection and six selected diamidine compounds.

Compound Identity	***Trypanosoma evansi* strains**						
	STIB 806K*	CAN86/ Brazil	Colombia	Kazakhstan	Philippines	RoTat 1.2	Vietnam
Standard drugs							
Suramin	87.6	44.0	59.8	61.0	54.0	76.2	78.4
Diminazene	12.5	12.2	13.3	13.1	12.2	12.6	12.5
Cymelarsan	1.1	1.2	1.2	1.2	1.3	1.3	1.2
Quinapyramine	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Diamidines							
DB 75	2.3	2.6	3.7	2.5	1.3	2.7	2.5
DB 820	5.4	4.7	4.1	5.6	3.4	4.6	4.1
DB 867	1.7	3.0	1.9	2.3	1.3	1.5	1.5
DB 930	4.3	4.2	6.5	5.7	10.7	4.1	3.9
DB 1192	10.5	9.6	8.2	10.7	10.4	9.9	10.3
DB 1283	6.5	5.9	5.6	6.3	8.6	6.5	5.9

* The STIB 806K strain represents the reference *T. evansi* strain in this study.

For the six diamidine compounds tested, the IC_{50} values of each compound (from each different strain), was compared with the IC_{50} value obtained using the reference STIB 806K strain. The strains Colombia, Kazakhstan, RoTat 1.2 (from Indonesia) and Vietnam, all

showed similar drug sensitivities to those seen with the Chinese reference strain. Slight variations were seen in the CAN86/Brazil strain with DB 867, where the IC_{50} value was approximately twice that of the reference strain and in the Philippines strain with DB 930, where the value was approximately two and a half times higher than that seen with STIB 806K. Overall, the *in vitro* strain sensitivity IC_{50} values were found to range between 1.3 and 10.7 ng/ml.

Discussion

The drug discovery and development pipeline expresses the various selective parameters which are required to be met in order to produce an effective and marketable drug. By screening large numbers of compounds, it is possible to select only those compounds presenting the given criteria important for an ideal drug. In this case, this study has enabled the screening of 181 novel diamidine compounds to be positively selected for compounds exhibiting high anti-trypanosomal activity against *Trypanosoma evansi* infection, reduced potential for drug resistance, low cytotoxicity against mammalian cells and of course low or no toxicity involved in an *in vivo* mouse system.

The four standard drugs available for the treatment of *T. evansi* infection (suramin, diminazene, cymelarsan and quinapyramine) are inadequate in controlling the disease. An alternative compound for *T. evansi* infection should exhibit improved or greater activity against the pathogen than the standard drugs can currently provide. The selective IC_{50} cut-off point for this criterion therefore lies below 12 ng/ml. The 181 novel diamidine compounds tested in this study against *T. evansi* all produced inhibitory concentration (IC_{50}) values less than 30 ng/ml, whilst 129 of these compounds had IC_{50} values between 1.2 ng/ml and 10 ng/ml. It is evident from these results that these dicationic molecules are highly active against *T. evansi in vitro*. As was originally hypothesised, diamidine compounds found to be highly active against the human pathogenic *T. b. rhodesiense* strain are also highly effective against animal pathogenic *T. evansi* strains.

To some extent, drug resistance has overcome any beneficial efficacy presented by the current drugs, especially suramin. By testing the novel diamidine compounds against a genetic knock out strain (TbAT1) lacking the gene encoding for the nucleoside P2 transporter, diamidine compounds relying mainly on this mode of action can be identified. An elevated

IC_{50} value, compared to the resulting IC_{50} value obtained for that compound against the respective trypanosome strain, demonstrates reduced trypanocidal activity due to a net reduction in drug uptake. The selective IC_{50} cut-off point for this criterion is thus placed at less than 20 ng/ml. Since drug resistance against dicationic diamidines tends to occur largely due to a lack in functional P2 transportation, any compounds demonstrating an elevated IC_{50} value against the TbAT1 strain also reveal a susceptibility to potential drug resistance. In a wider context, such a compound would not achieve the advantages sought after in a new anti-trypanosomal agent against *T. evansi* infection.

As well as possessing high activity and reduced potential for developing drug resistance, the ideal anti-trypanosomal agent should also exhibit limited toxicity. Cymelarsan and quinapyramine both appear to be hindered through toxicity. Although both standard drugs are highly active against *T. evansi*, the toxicity these drugs display remains a major issue in their chemotherapeutic use. The *in vitro* cell cytotoxicity assay, together with the preliminary *in vivo* toxicity tests performed using NMRI mice, could be used in this study to eliminate compounds, which revealed harmful effects on either mammalian cells alone or within a living organism, in this case a mouse model. For the cytotoxicity criterion against mammalian (L6) cells, the selective cut-off point includes any compound demonstrating an IC_{50} value of greater than 90’000 ng/ml. As a new drug against *T. evansi* infection will most likely be administered to animals, the preliminary *in vivo* toxicity tests could be considered as one of the most important criterions assessed in this study. Therefore, all diamidine compounds found to be lethal or toxic within the preliminary *in vivo* toxicity mouse experiments, are negatively selected for, leaving only the non-toxic diamidine compounds present to continue along the drug pipeline and warrant further investigation.

Although *in vivo* toxicity is an important criterion for drug discovery and development, another vital aspect in drug discovery is of course *in vivo* activity. Several of these compounds demonstrate high anti-trypanosomal activity *in vitro*, yet this activity is not necessarily displayed correspondingly when the compound is tested within a living organism. The next set of criteria to be investigated must essentially be performed *in vivo* and should include curative efficacy trials within a mouse model. For compounds that show no preliminary toxicity in the mouse, dose-response experiments could be carried out to determine primarily, the most active novel diamidine compounds and secondly, the lowest curative dose of those active compounds.

Based on the results obtained in this initial *in vitro* study, including the preliminary toxicity data derived from the mouse model, a total of sixty-three compounds have been found

eligible for continued investigation in an *in vivo* mouse model. Although the compound, DB 75 demonstrated toxicity at 50 mg/kg (i.p.) in the preliminary toxicity test and should not theoretically qualify for continued investigation, this compound will be included in all future experiments as an extra control compound, due to its current potential (together with its prodrug compound, DB 289) as a candidate drug against human sleeping sickness.

An additional criterion investigated in this initial *in vitro* diamidine study was strain sensitivity. Six diamidine compounds were selected for *in vitro* strain sensitivity testing, due to their curative efficacy determined within an infected mouse model. The data of this study can be viewed in the next chapter. These six selected diamidine compounds, along with the four standard drugs, were thus first examined *in vitro* against seven different *T. evansi* strains. The STIB 806K strain (from China) acted as the reference strain, whilst the remaining six *T. evansi* strains were chosen on the basis of their geographical locations. This was done to determine whether dicationic compounds could demonstrate broader efficacy on separate strains of *T. evansi* found around the world. Although slight variations were seen in the CAN86/Brazil and Philippines strain, on the whole, the results depicted indicate that these six diamidine compounds are highly effective *in vitro* against all seven *T. evansi* strains tested. Moreover, all six compounds displayed greater activity than that of suramin and diminazene.

Together with the criteria made apparent through this initial study, an effective, non-toxic, economically designed diamidine compound could be produced as an alternative to the four standard drugs currently available against *T. evansi* infection.

Acknowledgments

The authors wish to express sincere appreciation towards James E Hall and Susan Jones for the excellent technical and organisational support provided throughout this study in relation to the in-depth knowledge of the compounds used and to Karin Gysin and Pascale Steiger for their expert assistance with animal maintenance.

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In vivo investigations of selected diamidine compounds against *Trypanosoma evansi* using a mouse model

Kirsten Gillingwater[1], David W Boykin[2], Richard R Tidwell[3] and Reto Brun[1]

[1]Parasite Chemotherapy, Swiss Tropical Institute, Socinstrasse 57, 4002 Basel, Switzerland
[2]Department of Chemistry, Georgia State University, Atlanta GA, United States of America
[3]Department of Pathology, University of North Carolina, Chapel Hill NC, United States of America

Abstract

Surra is an animal pathogenic protozoan infection, caused by *Trypanosoma evansi*, which develops into a fatal wasting disease. Control measures tend to rely on either treatment or prophylaxis. However, with the continuous emergence of drug resistance, this tactic is failing and the pressing need for new, alternative chemotherapeutic agents is becoming critical. With the introduction of novel aromatic diamidines, a new category of anti-trypanosomal drugs was discovered. Nevertheless, their efficacy within an *in vivo* mouse model and the prophylactic nature of these compounds remains unknown.

In total, 63 compounds were previously selected for eligibility for further *in vivo* investigations within a mouse model. However, only 48 of these selected compounds were ultimately tested, whilst fifteen compounds could not be investigated due to compound availability and synthesis. Six of the compounds were capable of curing *T. evansi* infected mice at drug doses as low as 0.5 mg/kg and 0.25 mg/kg and were more effective than the standard drugs, suramin, diminazene and quinapyramine. Moreover, three novel diamidines were then tested *in vivo* against various *T. evansi* strains to determine strain sensitivity. Strains isolated from China, the Canary Islands and Colombia demonstrated a 100 % cure rate with all three compounds at 0.5 mg/kg. In addition, the preliminary prophylactic activity of diamidines was examined, using doses of 3 mg/kg and 10 mg/kg of DB 75 (2,5-bis(4-amidinophenyl)furan), which promoted 100 % survival of mice given prophylaxis up to six days before initial infection.

After all selective criteria were applied only three diamidine compounds qualified as lead compounds, considered to have the potential to act as clinical candidates against *T. evansi* infection. These three compounds, DB 75, DB 867 and DB 1192, warrant further investigation in a larger animal model.

Keywords: *Trypanosoma evansi*, diamidines, *in vivo*, dose-response, prophylaxis, mouse model, chemotherapy.

Introduction

Surra is an animal pathogenic protozoan infection, caused by *Trypanosoma evansi*, which develops into a fatal wasting disease. It is mechanically transmitted by biting flies of the genera, *Tabanus*, *Stomoxys* and *Lyperosia*. No intermediate hosts and no insect developmental cycles have as yet been identified (*Foil, 1989*). Control measures for protozoan diseases, such as Surra, tend to rely on either treatment or prophylaxis. Treatment usually involves the use of anti-parasitic drugs, whereas prophylaxis can usually be accomplished by drugs or sometimes through vaccination. Whether "prevention is better than cure" depends on the parasite being dealt with, the type of drugs available, the occurrence and severity of the disease and the economics obtainable in such a situation (*Ryley, 1982*).

In the case of Surra, drugs containing anti-parasitic properties are usually administered once an infection has already established itself, in the hope that such activity will render the parasite non-viable and the infection will clear (*Davila and Silva, 2000*; *Seidl et al., 2001*). However, with the continuous emergence of drug resistance, this tactic is failing and the pressing need for new, alternative chemotherapeutic agents is becoming critical (*Touratier, 2000*; *Reid, 2002*). Moreover, the prospect of a vaccination against *T. evansi* infection appears non-existent, for which a large proportion of responsibility can be placed upon the phenomenon of antigenic variability of trypanosomes in general.

With the introduction of novel aromatic diamidines, a new category of anti-trypanosomal drugs was discovered (*Francesconi et al., 1999*; *Wilson et al., 2005*). Several of these dicationic compounds have shown great efficacy *in vitro* against both human and animal pathogenic trypanosome species. A previous study into these diamidine compounds against *T. evansi* enabled the positive selection of diamidine compounds exhibiting high anti-trypanosomal activity, reduced potential for drug resistance, low cytotoxicity against mammalian cells and low or no toxicity involved within an *in vivo* mouse model. However, their efficacy within an *in vivo* mouse model and the prophylactic nature of these compounds remains unknown.

In order to find an effective, non-toxic, economically designed diamidine compound, which could act as an alternative to the four currently used drugs (suramin, diminazene, cymelarsan and quinapyramine), it is important to investigate the most active compounds and to determine their efficacy in the treatment of mice infected with *T. evansi*. Hence, the aim of this study was to investigate 63 previously selected diamidine compounds *in vivo* within a mouse model. The selected compounds were chosen based on their *in vitro* activity against a

Chinese *T. evansi* reference strain, a P2 transporter knock out trypanosome strain, their low cytotoxicity and low *in vivo* toxicity. The cut-off values for each of these criteria were IC_{50} values below 12 ng/ml, less than 20 ng/ml, greater than 90’000 ng/ml and not toxic at 100 mg/kg within a mouse model, respectively.

In response to the great efficacy produced by some of the diamidine compounds *in vitro*, several *T. evansi* strains isolated from various hosts and geographical locations were additionally tested *in vivo* using the mouse model. This was done to establish whether these compounds can provide curative efficacy against different *T. evansi* strains within living organisms. To explore any beneficial prophylactic properties contained within aromatic diamidines, one such compound called furamidine, or 2,5-bis(4-amidinophenyl)furan (also known as DB 75) (*Figure 4.1*), was further investigated, based on the high *in vitro* and *in vivo* activity seen by this compound against *T. evansi* strains. With curative doses of just 0.2 mg/kg, DB 75 was used in a prophylactic mouse experiment to determine its ability to cure infected mice before infection as well as after infection.

Figure 4.1. The chemical structure of 2,5-bis(4-amidinophenyl)furan (DB 75).

Materials and methods

Trypanosome strains

The reference STIB 806K strain was used for all *in vivo* experiments performed in this study. STIB 806K is a Chinese kinetoplastic *Trypanosoma evansi* strain, isolated from a water buffalo, by Lun in 1983. This strain was cloned and adapted to axenic culture conditions according to *Baltz et al.* (*1985*). In addition to the reference trypanosome strain, four further *T. evansi* strains, isolated from various geographical regions, were tested against a selection of diamidine compounds to determine *in vivo* strain sensitivity. Three of these strains (Colombia, Kazakhstan and Philippines) were originally obtained from the cryobank of the Institute of

Tropical Medicine (ITM) in Antwerp, Belgium, whilst the fourth *T. evansi* (Canaries) strain was isolated from a dromedary camel in the Canary Islands, by Gillingwater in 2006. A fifth strain, designated as TbAT1 K.O., was furthermore included in the tests. This strain is a genetic knock out of a *T. b. brucei* strain (STIB 777S), which is missing the *TbAT1* gene encoding the nucleoside P2 transporter.

Mice

Female NMRI mice weighing between 22 - 25 g were used for the *in vivo* experiments. All mice were specific pathogen free (SPF) and were maintained in standard Macrolon type II cages, at 22 °C and with a relative humidity of 60 - 70 %. Water and pelleted food was provided for the mice *ad libitum*.

Standard trypanocidal drugs

Suramin (Germanin® from Bayer, Leverkusen, Germany), diminazene aceturate (D-7770, Sigma, St Louis, MO, USA), cymelarsan (MelCy®, Rhône Mérieux, Toulouse, France) and quinapyramine sulphate (Trypacide®, May & Baker, Lagos, Nigeria) were used as the standard drugs in this study.

Diamidine compounds

The diamidine compounds were originally designed and synthesised by the chemists, David Boykin and Richard Tidwell. For the *in vivo* experiments, the diamidine compounds were selected by means of their previously demonstrated *in vitro* activity against *T. evansi* strains and *in vivo* toxicity, which was determined in an earlier study, the data of which can be viewed in the previous chapter.

Culture medium

Bloodstream form trypanosomes were cultivated in Minimum Essential Medium (MEM) (powder, GIBCO/BRL, No: 11400-033) with Earle's salts, supplemented with 25 mM HEPES, 1 g/l additional glucose, 2.2 g/l $NaHCO_3$ and 10 ml/l MEM non-essential amino acids (50x concentration). The medium was then further supplemented by adding 1 % of a 2-

mercaptoethanol stock (14 µl of 12 mM 2-mercaptoethanol was diluted in 10 ml of distilled water), 1 % of a stock consisting of 100 mM sodium pyruvate and 50 mM hypoxanthine and 15 % heat inactivated horse serum, according to *Baltz et al.* (*1985*). The complete medium is called Baltz MEM (BMEM).

Stock solutions and dilutions

A 1.5 mg amount of each compound was weighed out in powder form and dissolved in 15 ml of sterile distilled water, to provide a 10 ml/kg bodyweight mouse stock solution. From these stock solutions, further compound dilutions were made if necessary, depending on the compound dose being tested. These compound dilutions were then used for the *in vivo* experiments. Additionally, a 15 mg amount of the diamidine compound, 2,5-bis(4-amidinophenyl)furan (DB 75) was weighed out in powder form and dissolved in 15 ml of sterile distilled water, to provide a 100 ml/kg bodyweight mouse stock solution. From this stock solution, further compound dilutions were made for use in the *in vivo* prophylaxis experiment. Stock solutions and compound dilutions were made fresh on the day of administration and for each experimental procedure.

In vivo experiments

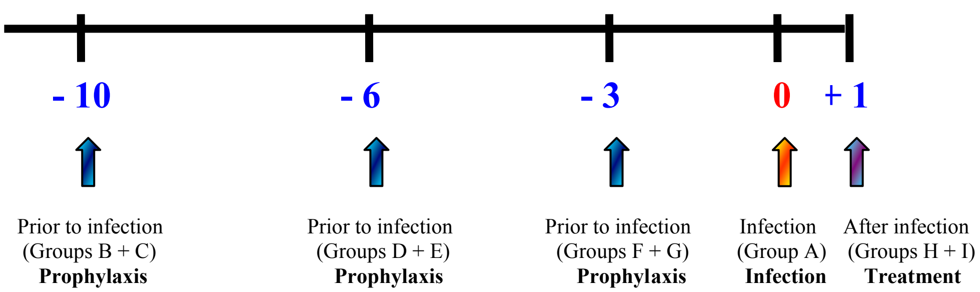
Female NMRI mice were divided into groups, each containing four mice. The mice were then infected with 10^4 parasites in 0.25 ml, from the STIB 806K *T. evansi* reference strain, using an intra-peritoneal (i.p.) route of infection. A parasitaemia was allowed to establish, before treatment was given (also using an i.p. route) on the following consecutive days: days 3, 4, 5 and 6 post-infection. Thereafter, the parasitaemia was checked twice a week using a tail blood examination technique, until day 60 post-infection. A two month experimental follow up period is used to account for any possible relapses, which may occur for inactive compounds. Subsequently, any surviving aparasitaemic mice are thus considered cured.

A similar experimental procedure was followed for the *in vivo* strain sensitivity tests, with the exception that the mice were infected with 10^4 parasites in 0.25 ml from either the respective *T. evansi* strains (Canaries, Colombia, Kazakhstan, Philippines or STIB 806K) or from the knock out *T. b. brucei* strain (TbAT1).

Prophylactic in vivo experiment

Thirty six mice were divided up into nine groups, each group consisting of four mice. The mice were then injected via an intra-peritoneal route (i.p.) with the respective DB 75 compound dose (3 mg/kg or 10 mg/kg), according to the respective day, prior to or after infection, depending on the group (*Figure 4.2*).

Figure 4.2. Time chart to show the number of days prior to infection (- #) and after infection (+ #) and the classification of the nine groups of mice (Groups A - I)



The volume of DB 75 given was dependent on the weight of each respective mouse, which was measured on the day of compound administration. On day 0 of the experiment, all nine groups of mice were infected with the STIB 806K strain. The mice were infected with 10^4 parasites in 0.25 ml, using an i.p. route of infection. Subsequently, the parasitaemia was checked twice a week using a tail blood examination technique, until day 60 post-infection. Thereafter, any surviving aparasitaemic mice were considered cured. The experimental procedure was independently repeated a second time, using the same criteria and conditions.

Results

In total, the four standard drugs currently in use against *Trypanosoma evansi* infection and forty-eight selected novel diamidine compounds were tested *in vivo* within this study. Although 63 compounds were originally selected to qualify for *in vivo* testing within a mouse model, fifteen of these compounds were not obtainable for testing due to compound availability and production. Therefore, only forty-eight compounds and an extra control compound (DB 75) were ultimately tested in the *in vivo* mouse model.

The results of the *in vivo* investigations for the four standard drugs can be seen in *Table 4.1*. At a drug dose of 1 mg/kg, suramin was able to cure 4/4 mice for the required length of the experiment (>60 days). However, as the dose of suramin was reduced, the survival of the mice decreased to just 3/4 and finally to 0/4 at 0.5 mg/kg and 0.25 mg/kg, respectively. In comparison, the standard diamidine drug, diminazene, was only capable of curing 3/4 mice at a 1 mg/kg dose. Twice this amount (2 mg/kg) was actually required in order to achieve the lowest curative dose for diminazene. Cymelarsan demonstrated the highest efficacy with a lowest curative dose of just 0.0625 mg/kg. In contrast, this value is sixteen times lower than the curative doses of suramin and quinapyramine, as well as being thirty-two times lower than the curative dose of diminazene. With a further reduction in the dose for cymelarsan, only a quarter of the mice could survive at 0.03125 mg/kg. A similar pattern (to that of suramin) was observed with the standard drug quinapyramine, where a 1 mg/kg dose was able to cure 4/4 mice, yet when reduced to 0.5 mg/kg and 0.25 mg/kg, only 3/4 and 1/4 mice were cured, respectively.

Table 4.1. *In vivo* investigations using the four standard drugs, against NMRI mice infected with *T. evansi* (STIB 806K), showing the number of mice cured over the number originally infected with the average survival of the mice (in days) depending on the drug dose, given four times (in mg/kg). The chemical families and structures of the drugs tested are also depicted.

Drug identity	Chemical family	Dose tested (mg/kg x 4)	Cured / Infected	Survival (days)	Chemical structure
Suramin	Naphtha-lene	**1**	**4/4**	**>60***	
		0.5	3/4	>51	
		0.25	0/4	11	
Diminazene	Triazene	**2**	**4/4**	**>60***	
		1	3/4	>44	
		0.2	0/4	9	
Cymelarsan	Melamino-phenyl arsenical	1	4/4	>60*	
		0.5	4/4	>60*	
		0.25	4/4	>60*	
		0.125	4/4	>60*	
		0.0625	**4/4**	**>60***	
		0.03125	1/4	>37	
Quinapyramine	Quinoline pyrimidine	**1**	**4/4**	**>60***	
		0.5	3/4	>57	
		0.25	1/4	>41	

* denotes all mice were cured at this dose (in **bold**, denotes the lowest curative dose of that drug)

In summary, thirty-eight selected diamidine compounds, designed and synthesised by the chemist, David Boykin, were investigated for *in vivo* activity within a mouse model. The

extra standard control compound, DB 75, was also included. The various compound doses tested, showing the number of mice cured over the number originally infected and the average survival of the mice (in days), can be seen in *Table 4.2*. All thirty-eight diamidine compounds were selected based on their ability to produce *in vitro* activity against *T. evansi* strains and a knock out *T. b. brucei* strain, below 12 ng/ml and 20 ng/ml, respectively. Furthermore, all thirty-eight compounds had previously demonstrated no significant cytotoxicity against mammalian cell lines and more importantly, were not toxic when applied as accumulative compound doses in mice at 100 mg/kg, when an intra-peritoneal route of administration was used.

Table 4.2. *In vivo* investigations using diamidine compounds, synthesised by the chemist, David Boykin, against NMRI mice infected with *T. evansi* (STIB 806K), showing the number of mice cured over the number originally infected with the average survival of the mice (in days) depending on the compound dose, given four times (in mg/kg). The chemical families and structures of the compounds tested are also depicted.

Compound identity	Chemical family	Dose tested (mg/kg x 4)	Cured / Infected	Survival (days)	Chemical structure
DB 75	Diphenyl furan	1	4/4	>60*	
		0.5	4/4	>60*	
		0.25	4/4	>60*	
		0.20	**4/4**	**>60***	
		0.125	3/4	>55	
		0.0625	1/4	>29	
DB 211	Pyrimidine	0.5	0/4	15	
DB 320	Pyrrole	1	3/4	>49	
DB 690	Diphenyl furan	**1**	**4/4**	**>60***	
		0.5	3/4	>57	
		0.25	1/4	>41	
DB 820	Aza-furan	1	4/4	>60*	
		0.25	**4/4**	**>60***	
		0.125	2/4	>50	
DB 828	Diaryl furan	1	0/4	23	
DB 850	Benz-imidazole	0.5	0/4	16	
DB 853	Benz-imidazole	1	0/4	18	
DB 867	Aza-furan	1	4/4	>60*	
		0.5	**4/4**	**>60***	
		0.25	3/4	>54	
		0.125	2/4	>46	

***Table 4.2.* continued…**

DB 877	Biphenyl	1 0.2	1/4 0/4	>35 12	
DB 930	Diphenyl furan	1 **0.5** 0.25 0.125	4/4 4/4 1/4 1/4	>60* **>60*** >38 >32	
DB 989	Benz-imidazole	0.5	0/4	27	
DB 1012	Diaryl furan	1	0/4	13	
DB 1017	Diphenyl furan	0.5	1/4	>43	
DB 1019	Biphenyl	1	1/4	28	
DB 1044	Diaryl pyridine	0.5	0/4	16	
DB 1046	Benz-imidazole	1	0/4	11	
DB 1052	Thiazole	1	3/4	>53	
DB 1055	Benz-imidazole	0.5	0/4	12	
DB 1152	Cinnamide	1	0/4	20	Structure not shown due to patent reasons
DB 1171	Indole	0.5	1/4	>30	
DB 1172	Indole	1	1/4	>39	
DB 1173	Diaryl furan	0.5	0/4	15	
DB 1177	Biphenyl	0.5	0/4	13	
DB 1191	Indole	0.5	0/4	7	
DB 1192	Indole	1 **0.5** 0.25 0.125	4/4 **4/4** 2/4 1/4	>60* **>60*** >51 >29	
DB 1194	Diaryl thiophene	0.5	0/4	16	Structure not shown due to patent reasons
DB 1213	Diaryl seleno-phene	0.5	0/4	27	Structure not shown due to patent reasons
DB 1220	Terphenyl	0.5	0/4	13	

Table 4.2. **continued…**

DB 1237	Aza-terphenyl	0.5 0.25	2/4 0/4	>47 16	
DB 1253	Aza-triaryl	0.5	0/4	7	
DB 1258	Aza-terphenyl	0.5	0/4	7	
DB 1272	Seleno-phene	0.5	0/4	18	Structure not shown due to patent reasons
DB 1283	Di-aza furamidine	1 **0.5** 0.25 0.125	4/4 **4/4** 2/4 0/4	>60* **>60*** >46 23	
DB 1288	Diaryl seleno-phene	0.5	0/4	23	Structure not shown due to patent reasons
DB 1340	Seleno-phene	0.5	0/4	8	Structure not shown due to patent reasons
DB 1342	Pyrrole	0.5	1/4	>34	
DB 1370	Aza-pyridine	0.5	0/4	21	
DB 1371	Aza-pyridine	0.5	0/4	14	

* denotes all mice were cured at this dose (in **bold**, denotes the lowest curative dose of that compound)

In vivo, six selected diamidine compounds, designed and synthesised by David Boykin, out of the thirty-eight originally tested, demonstrated very high efficacy within the mouse model. The following compounds, DB 867, DB 930, DB 1192 and DB 1283, produced lowest curative compound doses of 0.5 mg/kg for curing mice infected with a reference *T. evansi* strain. In addition, the compounds DB 820 and DB 75 produced lowest curative compound doses of 0.25 mg/kg and 0.20 mg/kg, respectively. At these relatively small amounts, 4/4 mice could survive for >60 days and all six compounds were more effective at curing the *T. evansi* infected mouse model, than the standard drugs, suramin, diminazene and quinapyramine. The lowest curative dose of cymelarsan (0.0625 mg/kg) remains four to eight times lower than the lowest curative doses of these six diamidine compounds. A new selective criterion was thus applied, whereby only compounds capable of curing *T. evansi* infected mice at doses of 0.5 mg/kg (or lower) will be positively selected for further investigation as lead compounds in a larger animal model.

Additionally, ten previously selected diamidine compounds, designed and synthesised by the chemist, Richard Tidwell, were also investigated for *in vivo* activity with a mouse model. The various compound doses tested, with the number of mice cured over the number originally infected together with the average survival of the mice (in days), can be seen in *Table 4.3*. All ten diamidine compounds were selected based on their ability to produce *in*

vitro activity against *T. evansi* strains and a knock out *T. b. brucei* strain, below 12 ng/ml and 20 ng/ml, respectively. Furthermore, all ten compounds had previously demonstrated no significant cytotoxicity against mammalian cell lines and more importantly, were not toxic when applied as accumulative compound doses in mice at 100 mg/kg, when an intra-peritoneal route of administration was used.

Table 4.3. *In vivo* investigations using diamidine compounds, synthesised by the chemist, Richard Tidwell, against NMRI mice infected with *T. evansi* (STIB 806K), showing the number of mice cured over the number originally infected with the average survival of the mice (in days) depending on the compound dose, given four times (in mg/kg). The chemical families and structures of the compounds tested are also depicted.

Compound identity	Chemical family	Dose tested (mg/kg x 4)	Cured / Infected	Survival (days)	Chemical structure
3 KEG 083	Pentamidine analogue	1	0/4	29	
4 SAB 075	Isoxazole	1 0.5	2/4 0/4	>43 19	Structure not shown due to patent reasons
5 MAA 101	Pentamidine analogue	1	1/4	>29	
5 SMB 093	Isoxazole	0.5	0/4	20	Structure not shown due to patent reasons
8 SAB 066	Isoxazole	0.5	2/4	>38	Structure not shown due to patent reasons
9 SAB 021	Isoxazole	0.5	0/4	9	Structure not shown due to patent reasons
12 SMB 003	Isoxazole	0.5	0/4	8	Structure not shown due to patent reasons
16 DAP 095	Isoxazole	**1** 0.5	**4/4** 1/4	**>60*** >29	
19 DAP 021	Isoxazole	0.5	0/4	12	Structure not shown due to patent reasons
19 DAP 025	Naphthylene	**1** 0.5 0.25	**4/4** 3/4 1/4	**>60*** >56 >36	Structure not shown due to patent reasons

* denotes all mice were cured at this dose (in **bold**, denotes the lowest curative dose of that compound)

Only two of the selected ten diamidine compounds demonstrated high *in vivo* efficacy against *T. evansi* infected mice. These two compounds, 16 DAP 095 and 19 DAP 025, are capable of curing 4/4 mice infected with *T. evansi* at 1 mg/kg doses. Both these compounds show better efficacy in the mouse model than the standard drug, diminazene. However, the cut-off value for the *in vivo* selective criterion is curative efficacy of *T. evansi* infected mice at

0.5 mg/kg doses. Since an alternative compound against *T. evansi* infection needs to show greater efficacy *in vivo* than the standard drugs, none of the diamidine compounds, designed and synthesised by the chemist Richard Tidwell, qualify for further *in vivo* investigation, based on this stringent criterion.

Taking into account the selective criteria applied during the *in vitro* study (detailed in the previous chapter) and the selective criteria from this *in vivo* mouse model, only six diamidine compounds currently qualify for further investigation in a larger animal model. These compounds are DB 75, DB 820, DB 867, DB 930, DB 1192 and DB 1283. Given that an alternative compound against *T. evansi* infection would significantly benefit many communities within developing countries, an economical compound would be an ideal candidate. Therefore, an element of cost-effectiveness was applied as another selective criterion, to establish which compounds contain the most advantageous properties for large-scale production. Any compounds, which require expensive raw materials or chemical components, as well as compounds involving complex and numerous synthetic steps, would in turn increase the cost of manufacture, hence the overall cost of the marketable compound. As a result, these compounds (DB 930 and DB 1283) were not selected for continued investigation.

The aza-furan compound, DB 820, is currently under investigation as a potential clinical candidate for human trypanosomiasis. Consequently, DB 820 was not selected for continued investigation against *T. evansi* infection (which is only animal pathogenic), due to this compound having already been selected for continued investigation for human sleeping sickness. Although DB 75 is in a similar situation to that of DB 820, this compound (DB 75) was nevertheless selected for continued investigation against *T. evansi* as an extra standard control diamidine. Together with DB 75, the remaining compounds DB 867 and DB 1192, qualified for *in vivo* testing within a larger animal model.

Three lead diamidine compounds (DB 75, DB 867 and DB 1192) were thus additionally tested *in vivo* against different *T. evansi* strains to determine strain sensitivity. The results obtained for the *in vivo* strain sensitivity mice experiments, for DB 75, DB 867 and DB 1192, can be seen in *Table 4.4*. These three compounds were administered in the mice at 0.5 mg/kg doses only, since this was the selective cut-off value for the *in vivo* mouse model criterion used for the reference STIB 806K *T. evansi* strain.

The *T. evansi* strains from the Canaries and Colombia demonstrated that all three compounds are capable of curing 4/4 mice at 0.5 mg/kg, which shows a similar pattern to that of the reference STIB 806K strain (marked in blue). The compound DB 75 was able to cure

4/4 mice infected with the Kazakhstan strain, unlike DB 867 and DB 1192, which were only able to cure 2/4 and ¼ mice, respectively, at the same dose. None of the compounds were able to provide complete survival of the mice, when they were infected with the Philippines strain, with the exception of DB 75, which managed to cure 2/4 mice only.

Table 4.4. *In vivo* strain sensitivity tests using three selected diamidine compounds (DB 75, DB 867 and DB 1192), against NMRI mice infected with either one of the five different *T. evansi* strains, isolated from various geographical locations, or the genetic knock out *T. b. brucei* strain (TbAT1 K.O.), showing the number of mice cured over the number originally infected with the average survival of the mice (in days), when each compound was tested against each strain, using a specific compound dose (in mg/kg) given four times.

Strain Identity	**DB 75**			**DB 867**			**DB 1192**		
	Dose tested mg/kg x 4	Cured / Infected	Average survival (days)	Dose tested mg/kg x 4	Cured / Infected	Average survival (days)	Dose tested mg/kg x 4	Cured / Infected	Average survival (days)
***T. evansi* strains**									
Canaries	0.5	4/4	>60*	0.5	4/4	>60*	0.5	4/4	>60*
Colombia	0.5	4/4	>60*	0.5	4/4	>60*	0.5	4/4	>60*
Kazakhstan	0.5	4/4	>60*	0.5	2/4	>47	0.5	1/4	>45
Philippines	0.5	2/4	>47	0.5	0/4	32	0.5	0/4	29
STIB 806K	0.5	4/4	>60*	0.5	4/4	>60*	0.5	4/4	>60*
***T. b. brucei* strain**									
TbAT1 K.O.	0.5	1/4	>35	0.5	2/4	>45	0.5	3/4	>51

* denotes all mice were cured at this dose

A *Trypanosoma brucei brucei* knock out strain (TbAT1) was also included in this panel of *T. evansi* strains to determine the *in vivo* effect of the three compounds in relation to their uptake mechanism and mode of action. As can be seen in *Table 4.4*, the compound DB 75 only cured 1/4 mice, whereas DB 867 and DB 1192 were increasingly more effective, by curing 2/4 and 3/4 mice, infected with the *T. b. brucei* knock out strain.

The number of mice cured over the number originally infected and the average survival (in days) of the nine groups of NMRI mice used in the prophylactic study are shown in *Table 4.5*. Group A acted as a negative control and therefore was given neither DB 75 prophylaxis nor treatment. Group A demonstrates that without any preventative or curative measures, all mice die after an average of 5 days, when infected with the STIB 806K *T. evansi* strain. As a comparison, Groups H and I were given DB 75 treatment, one day after infection with STIB 806K. These two groups acted as a positive control to illustrate the fact

that DB 75 can cure infection with STIB 806K at both 3 mg/kg (Group H) and 10 mg/kg (Group I) concentrations. Hence, as *Table 4.5* clearly shows, 4/4 mice were cured in Groups H and I.

Table 4.5. The number of mice cured over the number originally infected with the average survival (in days) of six groups of NMRI mice (Groups B - G), given 3 or 10 mg/kg doses of DB 75 prophylaxis, at 10, 6 or 3 days prior to infection with a kinetoplastic *T. evansi* strain. Two groups of NMRI mice (Groups H - I, positive control) were treated with 3 or 10 mg/kg doses of DB 75 respectively, one day after infection, whilst one group (Group A, negative control) was given neither prophylaxis nor treatment.

Group	DB 75 dose tested (mg/kg)	Prophylaxis given (days)	Cured / Infected	Average survival (days)
A	n/a	n/a	0/4	5
B	3	- 10	2/4	>32.50
C	10	- 10	2/4	>32.50
D	3	- 6	4/4	>60
E	10	- 6	4/4	>60
F	3	- 3	4/4	>60
G	10	- 3	4/4	>60
H	3	+ 1	4/4	>60
I	10	+ 1	4/4	>60

n/a denotes “not applicable”

Groups B to G were given DB 75 as a prophylactic determinant, but at various time points before initial infection. Again two compound doses were used, namely 3 mg/kg and 10 mg/kg. Starting with the smallest time difference, Groups F and G were given DB 75 prophylaxis three days before infection. At both 3 mg/kg (Group F) and 10 mg/kg (Group G) doses, DB 75 was able to successfully protect against STIB 806K infection and a 100 % survival rate was seen for both groups. This encouraging result was also seen with Groups D (3 mg/kg) and E (10 mg/kg), when DB 75 was given six days before initial infection. The last two groups, Groups B and C were given DB 75 prophylaxis ten days prior to infection with STIB 806K. At this time period, the prophylactic efficacy of DB 75 was sufficient to protect only 2/4 mice in both groups. There was no difference seen between the higher and the lower DB 75 doses (3 mg/kg and 10 mg/kg, respectively) in terms of average survival of the mice in days (>32.50).

Discussion

The aim of this study was to investigate sixty-three selected diamidine compounds, found to be highly effective *in vitro* against the reference STIB 806K *Trypanosoma evansi* strain. These diamidine compounds were not only chosen due to their antitrypanosomal activity against *T. evansi* strains, but also due to their reduced potential for drug resistance, determined by previous activity against a genetic P2 transporter knock out strain (TbAT1 K.O.). The low cytotoxicity against mammalian cells and the absence of toxicity within an *in vivo* mouse model are also important criteria for the specific selection of these diamidine compounds. However, fifteen of these selected diamidine compounds were not tested within an *in vivo* mouse model, due to compound unavailability. By determining the *in vivo* chemotherapeutic efficacy of the remaining forty-eight compounds within an *in vivo* mouse model, it was possible to further select the most efficient, non-toxic compounds, which could act as alternatives to the four standard drugs currently in use.

Using an *in vivo* mouse model, the compounds could be tested and observed for their efficacy within a living system. Although the *in vitro* results for these forty-eight compounds demonstrated potent activity against the parasite, *T. evansi*, the ability of these compounds to exert this same efficacy within a living system, such as an animal model infected with *T. evansi* strains, is a completely different matter. Factors such as absorption and metabolism start to play a role, enabling changes within the animal, the parasite or even the compound to occur and hence totally alter the resultant effect.

The standard drugs were able to cure 4/4 mice at drug doses of 1 mg/kg (for suramin and quinapyramine), 2 mg/kg (for diminazene) and 0.0625 mg/kg (for cymelarsan). In this mouse model, the diamidine, diminazene, appears to have the least efficacy amongst all the standard drugs currently on the market. In contrast, cymelarsan demonstrates the greatest curative activity, yet the high costs of this drug coupled with its limited availability throughout the world, overshadow its beneficial properties.

From the forty-eight compounds tested, 87.5 % of them demonstrated no significant *in vivo* activity at a 0.5 mg/kg compound dose given over four consecutive days. This stringent selection for active compounds at such a low dose is due to the *in vivo* activity observed by the four standard drugs against *T. evansi*. Since the general aim of this study is to discover potential lead compounds possessing greater activity than the standard drugs, only the remaining six compounds (providing high activity at 0.5 mg/kg given over four consecutive days) can be positively selected for in this case. Therefore 42 of the 48 compounds tested are

now removed from the drug pipeline, which expresses the various selective parameters required to be met in order to produce an effective and marketable drug. These six compounds (DB 75, DB 820, DB 867, DB 930, DB 1192 and DB 1283) will in turn continue onto the next step within the drug pipeline to determine whether they fit the next set of criteria.

Another selection criterion for clinical candidate compounds capable of entering into clinical trials includes the cost assessment and large-scale production of a compound. It was recognized that some of the compounds included a lengthy and complicated synthesis and therefore for future large-scale production, this would pose problems of practicality and cost. Since the aim is to find an economical alternative, complicated synthetic steps would increase the overall costs of these compounds (in this case, DB 930 and DB 1283) and thus they were removed from the pipeline. In addition, any compound previously selected for (and currently undergoing) investigation against the human form of sleeping sickness (DB 820) was also removed from the pipeline.

A further aspect investigated in this study was strain sensitivity and the ability of three lead compounds (DB 75, DB 867 and DB 1192) to cure mice infected with other *T. evansi* strains, isolated from various geographical locations. All three compounds cured mice infected with strains from the Canary Islands, Colombia and the reference STIB 806K Chinese strain. The Kazakhstan strain could be cured by DB 75, whereas DB 867 could cure only half of the mice and DB 1192, only a quarter.

When compared to the genetic knock out (TbAT1 K.O.) strain, the following results were observed, whereby DB 75 cured only a quarter of the TbAT1 K.O.-infected mice, whilst DB 867 cured 2/4 mice and DB 1192, 3/4 mice. We know that DB 75 uptake relies on the P2 transporter, which is missing in the TbAT1 K.O. strain and hence with a limited uptake rate, DB 75 was probably not able to cure all the mice. This may imply that DB 867 relies only partly on P2 transportation and thus could effectively cure only half the infected mice. Likewise, DB 1192 may not rely greatly on P2 transportation and thus strains missing this transporter may still be effectively cured by this compound (3/4 mice were cured for TbAT1 K.O.). Whether different uptake transporters are involved and which ones these may indeed be, has not yet been completely investigated.

Previously, the diamidine compound DB 75 demonstrated excellent *in vitro* chemotherapeutic properties. It produced an IC_{50} value of 2.3 ng/ml, when it was tested in various *in vitro* cell viability assays against a Chinese *T. evansi* strain (STIB 806K). Furthermore, this low IC_{50} value was seen to extend across a global panel of *T. evansi* strains, ranging from South America (Colombia and Brazil) through to Asia (Kazakhstan) and the

South East (Philippines, Indonesia and Vietnam). *In vivo*, DB 75 is capable of treating (and curing) mice infected with the reference *T. evansi* strain (STIB 806K) at overall treatment doses as low as 0.8 mg/kg. Similar to these results, a total dose of 2 mg/kg of DB 75 has shown curative efficiency *in vivo* within a mouse model against other *T. evansi* strains, namely from Colombia, Kazakhstan and the Canary Islands. Using DB 75, this study also provided a preliminary insight into the prophylactic nature of such dicationic molecules. The results reveal that DB 75 can remain effective within mice for up to six days, before that mouse is infected with *T. evansi*. This effect is capable of providing the mouse with 100 % protection. If the time period is extended until ten days prior to infection, only 50 % of the mice can be protected against *T. evansi*. This is seen at both 10 mg/kg and 3 mg/kg compound concentrations. Protection in a larger animal however, may last much longer than the six days observed for mice.

The application of a prophylactic treatment against infection with *T. evansi* may not be the ideal situation for disease control. The financial costs involved in logistically moving from region to region, applying the prophylactic treatment and the labour time required, to carry out such a control programme, does not provide additional long term benefits than if only the infected animals were identified and respectively treated. To ensure continued success of such a prophylactic programme, constant maintenance and reapplication of the treatment would need to be pursued; a situation which is difficult to follow for a disease that can be (mechanically) transmitted with such ease in regions where meeting such highly economical demands would impart a continuous struggle on both communities and governments.

With the selective criteria from a previous *in vitro* study, several *in vivo* (mouse model) criteria could be applied to these compounds within this study, to again select only those which have the ideal characteristics sought after. The remaining compounds are capable of curing *T. evansi* infected mice at doses of 0.5 mg/kg, more effectively than the standard drugs, suramin, diminazene and quinapyramine. In addition, these selected compounds are cost-efficient in terms of production, synthesis and manufacture and are not currently undergoing examination as lead compounds against human sleeping sickness. Although DB 75 does not qualify for all the specified criteria, this diamidine compound is included in all investigations against *T. evansi* as an additional control standard. In summary, two selected diamidine compounds remain (DB 867 and DB 1192), which warrant continued investigation as potential clinical candidates against *T. evansi* infection in a larger animal model.

Acknowledgments

The authors extend their thanks to James E Hall and Susan Jones for providing outstanding detailed knowledge of the compound structures and properties. In addition, to the Institute of Tropical Medicine (ITM) in Antwerp, Belgium, for permitting the use of their *T. evansi* strains in this study and to Pascale Steiger and Karin Gysin for their expert help in animal maintenance throughout the experimental period of this study.

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Preliminary acute toxicity trials of active novel diamidine compounds in a goat model

Kirsten Gillingwater[1], Carlos Gutierrez[2], Arlene Bridges[3], Huali Wu[4] and Reto Brun[1]

[1]Parasite Chemotherapy, Swiss Tropical Institute, Socinstrasse 57, 4002 Basel, Switzerland
[2]Veterinary Faculty of the University of Las Palmas, 3416 Arucas, Canary Islands, Spain
[3]School of Pharmacy, University of North Carolina, Chapel Hill NC, United States of America
[4]Department of Pathology, University of North Carolina, Chapel Hill NC, United States of America

Abstract

Chemotherapy is often the main method of control used to cure or prevent diseases. Since drugs usually produce undesirable effects, as well as desirable effects, successful chemotherapy is reliant on achieving a specific balance between the two. To conquer some of the limitations and problems involved in successful drug therapy, the events and effects occurring after drug administration can be investigated through pharmacokinetics and pharmacodynamics. The object of infectious disease drug therapy is to produce and maintain a therapeutic response, long enough to eliminate the disease causing agents, yet short enough to minimise toxicity and adverse effects. Investigations into a group of novel diamidine compounds found several to be highly effective against the protozoan parasite, *Trypanosoma evansi*, which causes Surra, a wasting disease affecting many domestic and wild animals worldwide.

Initial toxicity profiles of three selected lead diamidine compounds (DB 75, DB 867 and DB 1192) were evaluated in a goat model at a single administered compound dose of 4 mg/kg and at multiple (four) compound administrations at 1 mg/kg, using an intramuscular route of application. A preliminary pharmacokinetic study was also included, whereby blood samples were taken at specific time points and then analysed using HPLC/MS-MS. Overall, no signs of acute toxicity were seen in any of the goats for either diamidine compound (DB 75, DB 867 or DB 1192). In conclusion, all three compounds are considered safe to be investigated further in *T. evansi* experimentally infected goats and to determine their curative efficacy within such a model.

Keywords: Toxicity; diamidines; goat model; *Trypanosoma evansi*; pharmacokinetics.

Introduction

Chemotherapy is often the main method of control used to cure or prevent diseases. Since drugs usually produce undesirable effects, as well as desirable effects, successful chemotherapy is reliant on achieving a specific balance between the two. It is important to realise that the extent of therapeutic and toxic responses are functions of the dose given and that the effect of drug therapy will decrease with time after a single drug dose has been

administered. Indeed, continuous drug administration can lead to increased costs, in terms of adverse effects, economics and toxicity, implying that the balance between all necessary factors is difficult to attain. To conquer some of the limitations and problems involved in successful drug therapy, the events and effects occurring after drug administration can be investigated through pharmacokinetics and pharmacodynamics.

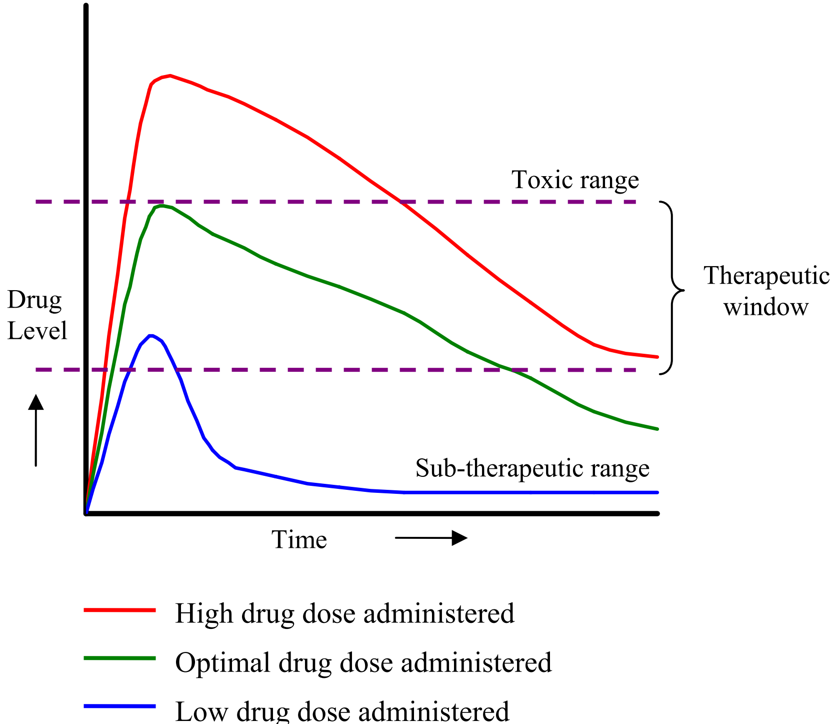
When a drug is administered, the rate at which it initially enters the body is greater than its rate of elimination. The concentration of the drug increases in the blood and in the tissues to gain the desired therapeutic effects; on the other hand, it can sometimes produce toxicity. Eventually, the rate of elimination will surpass the rate of absorption and hence the concentration of the drug in the blood and tissues (and of course the therapeutic effects) subsides. To apply drugs optimally, the mechanisms of absorption, distribution and elimination must be known, along with the kinetics included in such processes (*Rowland and Tozer, 1989*). The object of infectious disease drug therapy is to produce and maintain a therapeutic response, long enough to eliminate the disease causing agents, yet short enough to minimise toxicity and adverse effects. This important therapeutic window is illustrated in *Figure 5.1*, where if the drug dose administered is too high, toxicity may occur as the drug concentration in the body reaches the range in which adverse or toxic effects arise. Similarly, if the administered drug dose is too low, the period of time in which the required effective drug concentration remains within the therapeutic window may be too short, resulting in unproductive sub-therapeutic levels and unsuccessful treatment. The optimal drug dose to be administered is therefore a balance between the amount of drug present in the body and the length of time it remains there, before it is eliminated.

A group of chemical compounds known as aromatic diamidines bind to the minor groove of DNA and display a broad spectrum of anti-parasitic activity. Pentamidine has found a current use in the treatment of early stage African trypanosomiasis, antimony-resistant leishmaniasis and AIDS associated *Pneumocystis carinii* (*Boykin, 2002*). Nevertheless, the beneficial effects of pentamidine are hindered, due to its toxicity causing hypotension, dysglycaemia and renal and hepatic dysfunction (*Drake et al., 1985*). Chemically, it has been shown that pentamidine-related toxicity is partly due to the cleaving of the linker during drug metabolism (*Berger et al., 1992*).

With the introduction of synthalin, the first diamidine compound with anti-parasitic properties, a series of toxicity studies into such compounds was conducted within the 1920s (*Blatherwick et al., 1927*; *Bodo and Marks, 1928*). The ability of synthalin to produce hypoglycaemia, causing serious hepatic and renal damage, was considered to be derived from

malfunctions during normal glyconeogenesis. Synthalin caused a depletion of glycogen in the liver, hence leading to a breakdown of muscle glycogen and an increase in lactic acid within the blood. Many related compounds showed similar properties (*Broom, 1936*) and so, an attempt was eventually made to synthesise aromatic diamidines with improved properties and reduced toxicity (*Boykin, 2002*).

Figure 5.1. Diagrammatic sketch to show the therapeutic window within which the amount of administered drug dose (drug concentration within the body) and the length of time (the drug remains within that therapeutic window) is important to ensure safe and successful drug therapy.



Amongst these new and improved dicationic compounds, several were found to be highly effective against the protozoan parasite, *Trypanosoma evansi*, which causes Surra, a wasting disease affecting many domestic and wild animals worldwide (*Holland et al., 2004*; *Lun et al., 2004*). Since chemotherapy is the main form of control management for Surra, existing drugs (troubled with drug resistance and unacceptable toxicity) need to be replaced with new and improved compounds providing a more economical, effective and safer alternative for chemotherapeutic action against *T. evansi* (*El Rayah et al., 1999*).

Recent toxicity studies into these selected diamidine compounds have not yet been performed and before such compounds can enter clinical trials, the toxic effects of aromatic diamidines need to be fully examined. The compounds DB 867 and DB 1192 were previously

selected for their *in vitro* and *in vivo* activity (within a mouse model) against *T. evansi* strains, their reduced potential for drug resistance, as well as low cytotoxicity against mammalian cells and no preliminary toxicity in mice. In addition, these compounds were selected for further testing, due to their cost-effective manufacture as possible clinical candidates against *T. evansi* infection. The compound DB 75 has been included in these investigations as an extra standard control compound. The aim of this preliminary toxicity trial was therefore to determine whether the selected diamidine compounds, DB 75, DB 867 and DB 1192 produce signs of acute toxicity in goats, when administered at a single dose of 4 mg/kg or at accumulative multiple doses of 1 mg/kg. Should these compounds exert no toxic effects at the tested compound doses, it may be possible to formulate a safe and successful treatment schedule to determine the efficacy of these diamidines against *T. evansi* infected goats.

Materials and Methods

Study site and goats

The toxicity study took place within the Veterinary Hospital of the University of Las Palmas in Arucas, Gran Canaria in June 2006 and was approved by the ethical committee of the Hospital Clinicó Veterinario, Gran Canaria. Six female Canarian goats, weighing between 21 - 31 kg and no less than six months old were purchased from a local dairy farmer and then transported to the facility. Upon arrival, the goats were housed in enclosed pens and allowed to acclimatise to their new surroundings for one week. Thereafter, each goat was given a numbered ear tag, weighed and checked for current or previous infections. All six goats were confirmed to be clinically healthy at the start of the experiment. Throughout the experimental period, the goats were given food (pelleted nutrients, dried corn and hay) three times a day and water was provided *ad libitum*.

Selected diamidine compounds

The selected diamidine compounds were originally designed and synthesised by the chemist, David Boykin. The compounds DB 75, DB 867 and DB 1192 were used as the test compounds in these preliminary goat toxicity trials, based on their previous *in vitro* and *in*

vivo activity against *Trypanosoma evansi* and preliminary toxicity within a mouse model, which was assessed in previous chapters.

Stock solutions

A 160 mg amount of the compounds DB 75, DB 867 and DB 1192 were weighed out in powder form and dissolved in 4 ml of sterile distilled water with 10 % DMSO, to provide a 40 mg/ml stock solution. In addition, a 300 mg amount of the compounds DB 75, DB 867 and DB 1192 were weighed out in powder form and dissolved in 30 ml of sterile distilled water with 10 % DMSO, to provide a 10 mg/ml stock solution. These stock solutions were then applied in the goat toxicity trials as 10 kg/ml working solutions and were made fresh on the day of the first administration. Thereafter, the stock solutions were kept stored at 4 °C.

Toxicity trials

At the start of the toxicity trials, the goats were divided into three groups of two goats. Each group was allocated to one selected diamidine compound as shown in *Table 5.1*. One goat from each group primarily underwent a single toxicity trial, where a single compound application of 4 mg/kg was given, whilst thereafter both goats in each group undertook a multiple toxicity trial, whereby four compound applications of 1 mg/kg were given at two hour intervals, over a total six hour period. All compound applications were administered using an intramuscular route into the lower third region of the neck muscles. The goats were individually observed and assessed for signs of acute toxicity after every compound application given. Signs of acute toxicity monitored included tremors, lacrimation, excess salivation, irritation (scratching at injection site or general discomfort), excess urination, diarrhoea, hypertension and hypotension.

Blood samples (4 ml volumes) were taken for pharmacokinetic analysis from the goats at the following time points of 0.25, 0.50, 1, 2, 4, 8, 24 and 48 hours after the administration of the single 4 mg/kg compound application and at the following time points of 0.25, 0.50, 1, 2, 4, 8, 24, 48, 72 and 96 hours after the administration of the fourth and final compound application of 1 mg/kg for the multiple compound application toxicity trials. In addition, blood samples were taken from all six goats before the toxicity trials began, to determine control plasma levels, as well as just before each compound application of 1 mg/kg was given during the multiple toxicity trials, to determine the trough levels.

Table 5.1. The allocation of lead compounds with the goat numbers and weights (in kg) along with the compound doses given (in mg/kg) for either the single or multiple application toxicity trials.

Group	Number of goats	Lead compound	Goat number	Goat weight (kg)	Dose given (mg/kg)	Toxicity trial performed
1	2	DB 75	0823	27.0	4 1 (*x 4*)	single multiple
			0865	21.5	1 (*x 4*)	multiple
2	2	DB 867	0866	31.0	4 1 (*x 4*)	single multiple
			0850	21.0	1 (*x 4*)	multiple
3	2	DB 1192	0818	29.0	4 1 (*x 4*)	single multiple
			Grey	24.5	1 (*x 4*)	multiple

Blood sample collection

Blood samples were collected at regular time intervals from each of the goats using clean, sterile Vacutainer® (Becton Dickinson, Plymouth, UK) tubes of 4 ml volume, containing the anticoagulant, ethylene diamine tetra acetic acid (EDTA). The tubes were inverted several times, immediately after blood was taken from the jugular veins of the goats, to ensure equal distribution of the anticoagulant throughout the blood sample taken. The tubes were then kept on ice until they reached the laboratory for plasma preparation.

All blood samples taken at various time points, for either the single compound application or multiple compound application toxicity trials, were centrifuged at 3000 *g* for ten minutes. Subsequently, the plasma was removed and placed into sterile polypropylene cryotubes, before being stored away from light at -80 °C.

Plasma sample preparation

Plasma aliquots (25 µl volumes) were vortexed with 200 µl of a 12.5 nM internal standard (DB 103) dissolved in methanolic HCl (8:1 methanol: 1N HCl). Protein was precipitated by centrifugation (Beckman GS-6R) at 3000 rpm for 7 minutes at room temperature. The supernatant (120 µl) was then transferred to 96-well plates and evaporated under heated nitrogen (EVX Evaporator, Apricot Designs, USA). Thereafter, the samples were reconstituted in the plate, with 60 µl of high pressure liquid chromatography (HPLC) grade water (Fisher Scientific, USA), for analysis by mass spectrometry. Calibrators were prepared separately for each analyte. Stock solutions of DB 75, DB 867 and DB1192 were

prepared in water. Aqueous dilutions of each stock solution were prepared to provide the following spiking solutions: 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250 and 500 µM. To produce the intended calibration solutions of 5, 10, 25, 50, 100, 250, 500, 1000, 2500 and 5000 nM, 0.6 µl of aqueous spiking solution was added to 60 µl of untreated commercial goat plasma. These calibrators were then treated in the same way as the plasma samples. Curves were then prepared and analysed in duplicate, at both the beginning and the end of the analytical run.

Mass spectrometry analysis

Concentrations of DB 75, DB 867 and DB 1192 were determined by Liquid Chromatography/Triple Quadrupole Mass Spectrometry (HPLC/MS-MS). The HPLC/MS-MS system consisted of two solvent delivery pumps (Shimadzu Scientific, Colombia, MD, USA), a switching valve (Valco, Houston, TX, USA), a thermostated (6 °C) LEAP HTC auto-sampler (Carrboro, NC, USA) and an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA). Reversed-phase gradient chromatography was used to elute the compounds from an analytical column (Aquasil, C18 5 µm, 50 × 2.1 mm) at a flow rate of 0.75 ml / minute, following an 8 µl injection. Starting conditions for each injection included 95 % water with 0.1 % formic acid (v/v) and 5 % methanol with 0.7 % formic acid (v/v). This was held constant for 1 minute while the column eluted to waste. After 1 minute, the eluent was directed to the mass spectrometer and the organic phase (methanol with 0.1 % formic acid, v/v) increased linearly, reaching 90 % organic, 3.25 minutes post-injection. This was held for 0.5 minutes to wash the column. The column was then re-equilibrated to starting conditions for the final 1 minute. The total run time was 4.75 minutes.

The mass spectrometer was connected to the HPLC system by a TurboIon Spray® interface (MDS Sciex, San Francisco, CA, USA). Nitrogen, from a nitrogen generator (Peak Scientific, Bedford, MA, USA), was used as the curtain, nebulizer and collision gases. User controlled voltages, gas pressures and source temperature were optimised via direct infusion, for DB 103 (the internal standard), DB 75, DB 867 and DB 1192. All compounds were analysed in positive ion mode, using the following transitions preset in multiple reaction monitoring scans: DB 103 (internal standard) - 385.2 to 271.2, DB 75 - 305.2 to 288.2, DB 867 - 306.3 to 289.2 and DB 1192 - 292.3 to 275.1. The calibration curves were generated based on peak area ratios (analyte: internal standard) from 5 to 5000 nM. Curves for all analytes followed a linear $y = mx + b$ regression with a $1/x^2$ weighting. Typical r values were 0.997 or greater and accuracy was between 80 and 120 %. Automated sample acquisition and

data analysis was performed using Analyst software (version 1.4.1, Applied Biosystems, Foster City, CA, USA).

Pharmacokinetic parameters

The pharmacokinetic parameters were determined using Microsoft® Excel software, with a functional set of add-ins applied, together with a graphical programme (GraphPad Prism 5, GraphPad Software Inc, USA). Pharmacokinetic parameters measured and calculated included the peak concentration (C_{max}), time at which peak concentration occurs (T_{max}), the area under the curve (AUC), the area under the first moment curve (AUMC), the mean residence time (MRT), the half life ($t_{½}$) and the rate of elimination constant (K_{elim}). The MRT was calculated with the function, AUMC/AUC and the rate of elimination constant was obtained with the function, 1/MRT.

Results

The signs of acute toxicity seen in the goats for both the single and the multiple toxicity trials for the three selected lead compounds (DB 75, DB 867 and DB 1192) are shown in *Table 5.2*. None of the goats demonstrated any forms of hypotension or hypertension after application of the single or multiple injections for each of the three compounds. Similarly, no extreme lacrimation, tremors, excess urination or onset of diarrhoea were reported in any of the goats. All the goats demonstrated a slight excess in salivation, however, this was not considered to be directly related to the drug injections.

For the goats within group 1 (DB 75) and group 2 (DB 867), some irritation was observed for both the single and multiple toxicity trials, by means of scratching at the injection site. These goats additionally showed a short phase of uncomfortability, immediately after each compound injection was applied. Nevertheless, these symptoms disappeared after ten minutes and the goats returned to their normal behaviour.

Compound concentrations can be seen in *Figure 5.2* for the compound DB 75, *Figure 5.3* for the compound DB 867 and *Figure 5.4* for the compound DB 1192. The plasma samples taken were analysed by HPLC/MS-MS to determine the concentrations (in ng/ml) of each lead compound tested at each specific time point. From these values, concentration

versus time curves were plotted and the line of best fit applied to both the single and multiple compound application doses tested.

Table 5.2. Signs of acute toxicity expected after the i.m. application of three selected diamidine compounds (DB 75, DB 867 and DB 1192) in a single compound application (4 mg/kg) and a multiple compound application (4 x 1 mg/kg) toxicity trial. The presence or absence of each sign is depicted as a (✓) or as a (x), respectively.

Group	Lead compound	Toxicity trial	Goat number	Signs of acute toxicity*							
				Hypo	Hyper	(L)	(S)	(Tr)	(Ir)	(U)	(D)
1	DB 75	single	0823	x	x	x	x	x	✓	x	x
		multiple		x	x	x	x	x	✓	x	x
		multiple	0865	x	x	x	x	x	✓	x	x
2	DB 867	single	0866	x	x	x	x	x	✓	x	x
		multiple		x	x	x	x	x	✓	x	x
		multiple	0850	x	x	x	x	x	✓	x	x
3	DB 1192	single	0818	x	x	x	x	x	x	x	x
		multiple		x	x	x	x	x	x	x	x
		multiple	Grey	x	x	x	x	x	x	x	x

***Signs of acute toxicity:** Hypo: *Hypotension*; Hyper: *Hypertension*; (L): *Lacrimation*; (S): *Salivation* (excess); (Tr): *Tremors*; (Ir): *Irritation*; (U): *Urination* (excess); (D): *Diarrhoea*.

For the lead compound DB 75, the peak concentration for both single and multiple trials occurred at the same time point (≤ 0.25 hours), yet was two-fold higher in the single toxicity trial (≥ 3690 ng/ml) compared to the multiple trial (≥ 1830 ng/ml). The area under the curve (AUC) was similar for both the single (159390) and multiple (138183) trials with DB 75, whereas the AUMC differed between the two by a factor of 1.7 (3'715'369 and 6'306'900, respectively). The MRT was twice as long in the multiple DB 75 trial and the elimination rate constant was twice as slow.

The peak concentration for the single toxicity trial using DB 867 occurred at ≥ 2880 ng/ml around ≤ 0.5 hours after the initial compound injection of 4 mg/kg. The multiple dose trial reached a peak concentration of ≥ 3390 ng/ml, at (≤) one hour after the fourth and final injection of 1 mg/kg DB 867. The AUC and the MRT for the multiple toxicity trial (300'997 and 47.1 hours) were approximately double that of the single toxicity trial (114'853 and 25.0 hours). The area under the first moment curve (AUMC) however, was five times greater in the multiple dose trial (14'162'688) than in the single (2'876'569). Similar to DB 75, the elimination rate constant of DB 867 was 0.04 for the single and 0.02 for the multiple toxicity

trials. The half life determined for the single toxicity trial with DB 867 was 120.5 hours, whilst the multiple toxicity trial produced a three-fold longer half life of 351.7 hours.

Figure 5.2. Compound concentrations in plasma samples taken from goats for DB 75, with the single (4 mg/kg) dose and after the last application of the multiple (4 x 1 mg/kg) dose, against time.

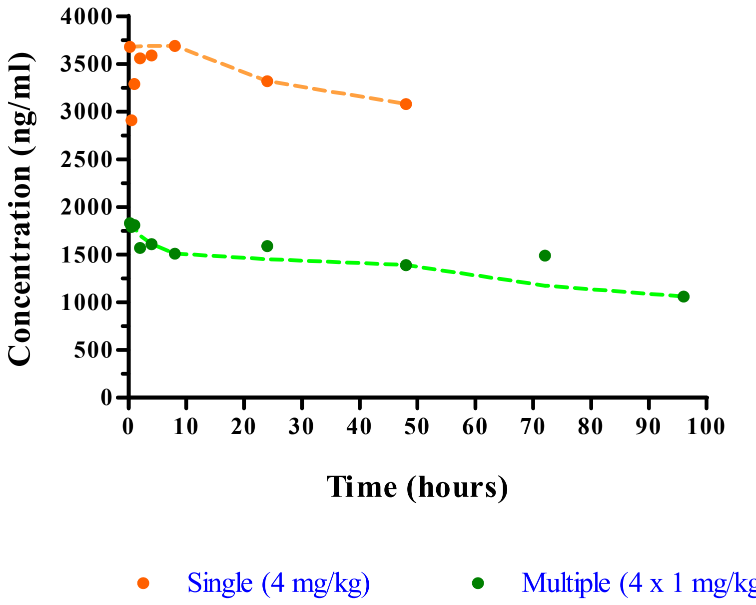


Figure 5.3. Compound concentrations in plasma samples taken from goats for DB 867, with the single (4 mg/kg) dose and after the last application of the multiple (4 x 1 mg/kg) dose, against time.

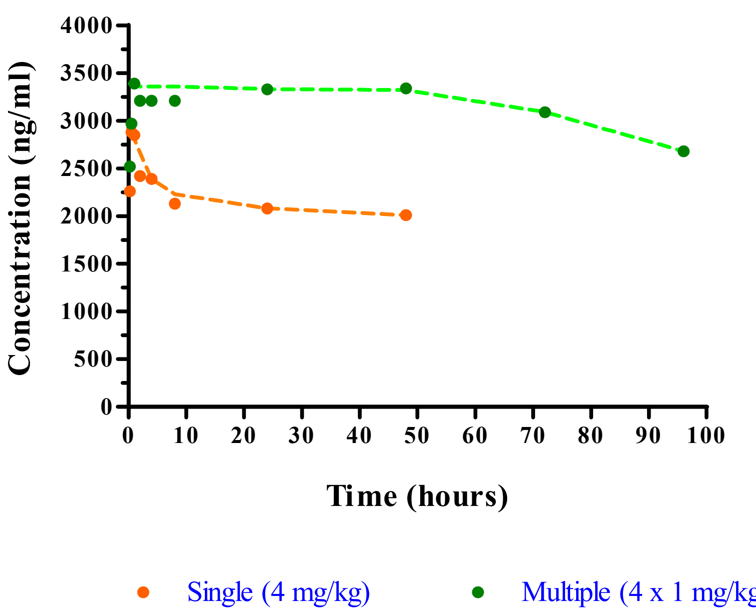
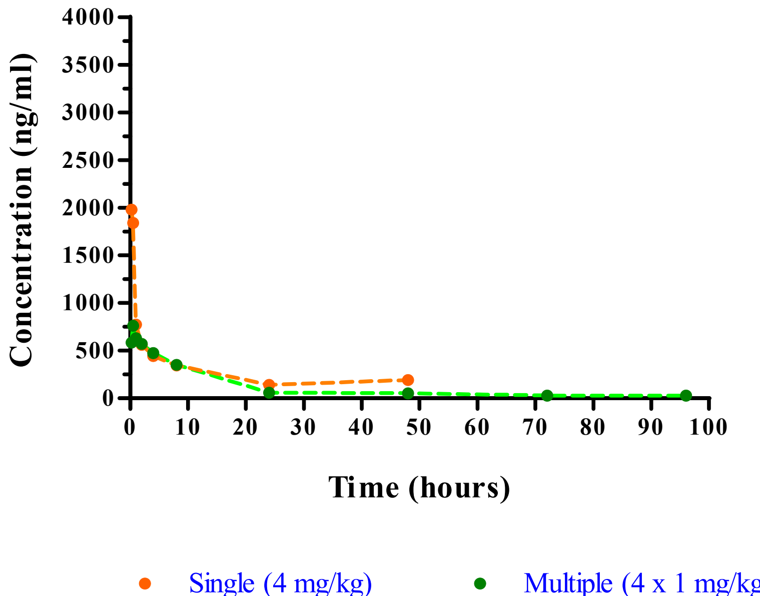


Figure 5.4. Compound concentrations in plasma samples taken from goats for DB 1192, with the single (4 mg/kg) dose and after the last application of the multiple (4 x 1 mg/kg) dose, against time.



The peak concentration of the single toxicity trial for DB 1192 (≥ 1980 ng/ml) was 2.5 times higher than the peak concentration of the multiple toxicity trial (≥ 762 ng/ml). In addition, the time taken for the peak concentration to occur was twice as long for the multiple dose trial (≤ 0.5 hours) as for the single DB 1192 dose trial (≤ 0.25 hours). Both the AUC and the AUMC were smaller in the multiple dose trial (10’093 and 207’593 compared to the single trial with 12’694 and 215’167), whereas the MRT differed by only 3.6 hours between the two toxicity trials. The elimination rate constant was 0.06 for the single trial and 0.05 for the multiple. The half lives for the single and multiple toxicity trials using DB 1192 were relatively similar at 16.7 hours and 19.0 hours, respectively.

Discussion

The aim of this preliminary toxicity trial was to determine whether any of the three selected lead diamidine compounds were toxic at 4 mg/kg, given as either a single dose or as four 1 mg/kg applications every two hours within healthy goats. No signs of acute toxicity were reported for any of the goats, given either a single 4 mg/kg injection or four multiple injections of 1 mg/kg for the lead compounds, DB 75, DB 867 and DB 1192. Irritation was

evident in the goats given both the single and multiple compound regimens for the lead compounds, DB 75 and DB 867. Coupled with a short phase of uncomfortability, it was established that these symptoms were due to the painful method of compound application (intramuscular) rather than to the lead compounds themselves.

Toxicity profiles were created based on the compound concentrations within plasma samples, taken from the goats at specific time points and then plotted against time, to give the concentration versus time curve. The compound concentrations established for DB 75 shows that both the single and multiple trials fall within the therapeutic range at 72 hours, with compound concentrations greater than 9.2 ng/ml. To remain within the therapeutic range, the minimal inhibitory concentration (MIC) for each compound must be exceeded for long enough to allow the compound to exert its biological activity. In this case, the MIC for DB 75 can be calculated as approximately four times the *in vitro* IC_{50} value of this lead compound at 72 hours against *Trypanosoma evansi*, based on previous *in vitro* experiments performed using cell viability assays (Chapter 3). Therefore, compound concentrations must exceed 9.2ng/ml, which in this case is four times the IC_{50} for DB 75. Since the shape of the curve declines very slowly for at least 100 hours, it appears that DB 75 remains well within the therapeutic range for the compound doses tested. The same is seen for the compound DB 867, which has an MIC at 72 hours of 6.8 ng/ml. Both the single and multiple trials have compound concentrations higher than the MIC at 72 hours and with the shape of the curve declining so slowly for at least 100 hours, the probability of DB 867 remaining within the therapeutic range is high.

The situation appears different for the third lead compound, DB 1192. With an MIC of 42 ng/ml at 72 hours, the toxicity profile for this diamidine compound shows that the chosen dose of 4 mg/kg may not be sufficiently high enough to remain within the therapeutic range. However, the therapeutic efficacy of DB 1192 will be revealed after this compound has been tested in an experimentally infected goat model.

In conclusion, all three selected lead diamidine compounds (DB 75, DB 867 and DB 1192) displayed no forms of acute toxicity in healthy goats and are thus considered safe for use in a *T. evansi* experimentally infected goat model to determine the efficacy of these compounds in a larger animal.

Acknowledgements

The authors wish to mention their appreciation for the hard work and effort invested by the staff of the Hospital Clinicó Veterinario in Arucas, Gran Canaria and for the kind use of their veterinary facilities. Additional thanks are extended to James E Hall for his assistance with the pharmacokinetic analysis.

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Efficacy study of novel diamidine compounds within an experimentally infected *Trypanosoma evansi* goat model

Kirsten Gillingwater[1], Carlos Gutierrez[2], Stijn Deborggraeve[3], Rosine Ali Ekangu[3] and Reto Brun[1]

[1]Parasite Chemotherapy, Swiss Tropical Institute, Socinstrasse 57, 4002 Basel, Switzerland
[2]Veterinary Faculty of the University of Las Palmas, 3416 Arucas, Canary Islands, Spain
[3]Department of Parasitology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium

Abstract

Trypanosoma evansi, the causative agent of Surra, presents as a fatal wasting disease in domestic and wild animals, found on the South American, African and Asian continents. Effective control methods are hindered with resistant strains, high costs and unavailable and toxic drugs. Alternative chemotherapeutic agents are urgently required, of which diamidines are showing promising results. Three lead compounds (DB 75, DB 867 and DB 1192) were investigated in an experimentally infected *T. evansi* goat model in the Canary Islands. Various diagnostic methods, including the haematocrit centrifugation technique (HCT), the card agglutination test for trypanosomes (CATT / *T. evansi*) and the polymerase chain reaction (PCR), were used to determine efficacy of the diamidines against *T. evansi* infection, where the PCR method was considered the gold-standard.

Complete curative efficacy of the experimentally infected goats was seen in the positive control group, treated with 5 mg/kg diminazene, and in the DB 75 and DB 867 groups, where goats were treated with 2.5 mg/kg of the respective diamidine compounds. In addition, complete cure was also observed in the lower dose group of DB 75 (1.25 mg/kg). The group treated with DB 867 at 1.25 mg/kg relapsed after four months follow up, post treatment. The diamidine compound DB 1192 was not as effective and treatment failures were observed in the goats after just seven days in the 2.5 mg/kg group, whilst the 1.25 mg/kg group provided no efficacy whatsoever. In conclusion, two diamidine compounds (DB 75 and DB 867) have presented better efficacy than the standard drug (diminazene) and could be considered as potential clinical candidates against *T. evansi* infection.

Keywords: *Trypanosoma evansi*, diamidines, goat model, chemotherapy, efficacy trial.

Introduction

Trypanosoma evansi is the causative agent of Surra, which presents itself as a fatal wasting disease in many domestic and wild animals, inhabiting the continents of South America, Africa and Asia (*Davila and Silva, 2000*; *Reid, 2002*; *Delafosse and Doutoum, 2004*). Through mechanical transmission performed by biting flies (Tabanids), infection can quickly spread amongst close-living herds of cattle, water buffalo, horses and camels (*Foil,*

1989). With the hindrance of resistant strains, unavailable and toxic drugs and the great economical losses involved, controlling this disease is a complex process (*Touratier, 2000*), which could be simplified if alternative chemotherapeutic agents were obtainable.

New diamidine analogues, one such alternative class of chemical compounds, were introduced (*Wilson et al., 2005*) and proved highly effective against *Trypanosoma brucei rhodesiense*, the causative agent of acute human sleeping sickness found mainly in Eastern Africa. As a comparison, these diamidine compounds were additionally tested against various *T. evansi* strains, yielding exceptionally good results in previously conducted experiments at an *in vitro* and *in vivo* (mouse model) level. The data for these experiments can be viewed in Chapter 3 and 4, respectively. Moreover, three selected diamidine compounds were examined for acute toxicity within a goat model, revealing promising prospects of diamidines as alternative chemotherapeutic representatives against Surra. To determine the potential of diamidines as clinical candidates against *T. evansi* infection, three selected lead compounds, DB 75, DB 867 and DB 1192 (*Figure 6.1*), were examined further to evaluate their ability to cure experimentally infected goats with *T. evansi*, in the Canary Islands, Spain.

Figure 6.1. The chemical structures of **a)** DB 867 and **b)** DB 1192

a)

HN NH NH_2 O N NH NH_2

b)

NH H_2N H_3C NH N H NH_2

T. evansi is currently endemic on the Canary Islands, originally due to the importation and subsequent diagnosis of a male dromedary camel in 1997, from the neighbouring area of West Africa, where Surra is highly prevalent. Camels were primarily imported to the Canary

Islands for labour purposes, but are nowadays principally raised for the tourism industry (*Gutierrez et al., 2005*). Seroprevalences in camels have been calculated to be around 4.8 % to 9 %, between 1997 and 1999 (*Gutierrez et al., 2000*). Although camels and horses are the major hosts affected on the Canary Islands, dissemination of the disease in other hosts has not yet been ruled out. Above all, goats may appear to play an important role in disease epidemiology, especially as potential reservoir hosts (*Gutierrez et al., 2004*).

Therefore, the aim of this study was to determine firstly whether goats could be inoculated with a local *T. evansi* strain (and provide a constant parasitaemia) and if so, whether these experimentally infected goats could be effectively treated with the three selected lead compounds, DB 75, DB 867 and DB 1192, at compound doses of 2.5 mg/kg and 1.25 mg/kg, given intramuscularly (i.m.). As a comparison, the standard drug, diminazene aceturate, would be included as a positive control compound, administered at 5 mg/kg i.m. Berenil® is the officially registered veterinary drug, yet it contains only 52.5 % of the active component, diminazene aceturate. A control group of goats receiving no compound treatment would also be established within the model. Thereafter, a five month follow up study is planned, to evaluate the curative efficacy of these compounds using various diagnostic methods. By means of such an efficacy study, potential new clinical candidates against *T. evansi* may be discovered.

Materials and methods

Parasite strain

A *Trypanosoma evansi* strain, designated as the Canaries (Rubio) strain, was the parasite strain employed for goat infection in this pilot “proof of concept” study. This strain was originally isolated by K Gillingwater in 2006, from a twenty-two year old dromedary camel, in a farm on Gran Canaria, belonging to the Canary Islands, Spain. The camel blood was immediately inoculated into mice and after a single mouse passage, was then used for experimental infection of the goats.

Mice

Female NMRI mice weighing between 25 - 30 g were used for strain isolation. All mice were specific pathogen free (SPF) and were maintained in standard Macrolon type II cages, at 22 °C and with a relative humidity of 60 - 70 %. Water and pelleted food was provided for the mice *ad libitum*.

Goats

This study was approved by the ethical committee of the Hospital Clinicó Veterinario, Gran Canaria. Twenty-seven female Canarian goats, weighing between 20 - 52 kg and older than 6 months, were purchased from a local dairy farmer. After transportation to the veterinary faculty, the goats were placed inside open pens and allowed to acclimatise for a week to their new surroundings. Thereafter, the goats were given numbered ear tags, weighed and checked for current or previous *T. evansi* infection using the diagnostic methods described within this study. Any goats showing positive results for previous *T. evansi* infection were removed from the project. The remaining goats were then divided into test groups, given oxytetracycline against nasal exudate and ivermectin as a standard anti-helminthic, before being placed into fly-proof pens. Throughout the study period, the goats were given food (pelleted nutrients, dried corn and hay), three times a day and water was provided *ad libitum*.

Standard trypanocidal compound

Diminazene aceturate (D-7770, Sigma, St Louis, MO, USA), was used as the standard positive control drug in this goat efficacy study. Berenil®, the official veterinary drug, contains only 52.5 % of the active component, diminazene aceturate.

Selected lead diamidine compounds

The selected lead diamidine compounds were originally designed and synthesised by the chemist, David Boykin. The compounds DB 75, DB 867 and DB 1192 were used as the test compounds in this goat efficacy study, based on their previous *in vitro* and *in vivo* activity and preliminary toxicity within a mouse model, together with preliminary toxicity results

obtained from a goat toxicity trial. The data for these previous investigations can be reviewed in chapters 3, 4 and 5, respectively.

Stock solutions

A 900 mg amount of the compounds, DB 75, DB 867 and DB 1192 were weighed out in powder form and dissolved in 36 ml of sterile distilled water with 10 % DMSO, to provide a 25 mg/ml stock solution. In addition, a 450 mg amount of the compounds, DB75, DB 867 and DB 1192 were weighed out in powder form and dissolved in 36 ml of sterile distilled water with 10 % DMSO, to provide a 12.5 mg/ml stock solution. A 1800 mg amount of the standard compound, diminazene aceturate was weighed out in powder form and dissolved in 36 ml of sterile distilled water with 10 % DMSO, to provide a 50 mg/ml stock solution. These stock solutions were then applied in the goat efficacy study as 10 kg/ml chemotherapeutic solutions and were made fresh on the day of the first administration. Thereafter, the stock solutions were kept stored at 4 °C.

Collection of blood samples

Blood samples were collected at regular time intervals from each of the goats within each of the test groups, using clean, sterile Vacutainer® (Becton Dickinson, Plymouth, UK) tubes (4 ml and 10 ml volumes), containing the anticoagulant, ethylene diamine tetra acetic acid (EDTA). The tubes were inverted several times, immediately after blood was taken from the jugular veins of the goats, to ensure equal distribution of the anticoagulant throughout the blood sample taken. The tubes were then placed on ice whilst diagnostic tests were carried out.

Haematocrit centrifugation technique

To analyse whether parasites were present or absent in the blood of the goats, the haematocrit centrifugation technique, adapted to field conditions (*Woo, 1970*), was utilised as the standard parasitological test for this efficacy study. A small amount of whole blood (70 μl), obtained directly after blood sampling of the goats, was taken up in a heparinised capillary and sealed with plasticine at one end. The capillary was then centrifuged for five

minutes at 9000 *g* and subsequently examined under light microscopy (x 10), for the presence of motile trypanosomes within the buffy coat layer.

CATT / T. evansi test

In addition, the CATT / *T. evansi* test was performed to act as a serological technique and determine the presence or absence of antibodies mounted specifically against *T. evansi* infections (*Bajyana Songa and Hamers, 1988*). Blood samples taken from the goats were centrifuged for ten minutes at 3000 *g*. All reagents, solutions and buffers required for the CATT test are already included within the commercially available kit (ITM, Antwerp, Belgium). Briefly, plasma dilutions were carried out, whereby 25 µl of goat plasma were mixed with 25 µl of buffer solution to give a ½ dilution. This step was then repeated to provide a ¼ plasma dilution, after which a 25 µl droplet was placed onto the corresponding circle on the card. Next, a drop (45 µl) of test reagent (antigen) was placed into the same circle and mixed carefully with a plastic spatula. The whole card was then placed onto a rotator and allowed to rotate for five minutes at seventy rotations per minute (rpm). Thereafter, the presence of agglutination within the circle determines a positive reaction. Furthermore, the positive reaction can be classified as strong (+++), intermediate (++), weak (+) or negative (-).

Polymerase chain reaction

As a validating molecular based technique, a PCR method (18S PCR for *Trypanozoon*) designed for the detection of parasite DNA was also applied. A 500 µl volume of whole goat blood was placed into a collection tube along with 500 µl of DNA stabilising buffer and mixed thoroughly. Thereafter, the PCR samples were stored at room temperature and in the dark, until they could be safely transported to the Institute of Tropical Medicine in Antwerp, Belgium, where the actual PCR procedures were completed. The PCR method was considered the overall gold-standard technique in this study.

DNA extraction for PCR

The DNA was extracted using the commercially available QIAamp® DNA blood mini kit from Qiagen® (Catalogue number: 51106, Qiagen®, Chatsworth, CA, USA). Briefly, 80 µl

of proteinase K (20 mg/µl) were added to the collection tube containing the goat blood sample. The sample was then incubated for 60 - 90 minutes at 58 °C and vortexed every 20 minutes. A 40 µl volume of lysing buffer was added, vortexed for 15 seconds to ensure complete mixing and quickly spun down. Thereafter the whole mixture was incubated for ten minutes at 70 °C. After incubation, 210 µl of pure ethanol were added, mixed again by vortexing and quickly spun down. The solution was placed onto a QIAamp® spin column and centrifuged for two minutes at 15’700 *g*, after which the filtrate was removed, since the DNA binds to the Si-membrane on the spin column. This spin column was then transferred into a new collection tube and 500 µl of washing buffer 1 were added, followed by centrifugation for two minutes at 5900 *g*. Again the filtrate was removed and the spin column transferred to a new collection tube. This time, 500 µl of washing buffer 2 were added and the mixture centrifuged for three minutes at 15’700 *g*. Once the filtrate had been removed, the spin column was placed inside a previously labelled collection tube and 50 µl of elution buffer were added. This tube was then incubated for one minute at room temperature, followed by a two minute centrifugation step at 5900 *g*, before finally storing the tube at -20 °C, until further testing with PCR amplification could be carried out.

PCR amplification

For the polymerase chain reaction (PCR), a 25 µl reaction solution contained 1 x PCR buffer (Qiagen, Hilden, Germany), 2.5 mM final concentration of magnesium chloride (Qiagen, Hilden, Germany), 200 µM of each dATP, dTTP, dGTP and dCTP (Roche, Mannheim, Germany), 0.1 mg/ml acetylated bovine serum albumin (Promega, Madison, Wis.), 0.8 µM of each primer, 1 unit of HotStar *Taq* polymerase (Qiagen, Hilden, Germany) and 2.5 µl of sample DNA. Double distilled water acted as the negative control and *Trypanosoma brucei* as the positive control in all PCR reactions performed. PCR amplification was carried out in 200 µl thin-wall PCR tubes (Abgene, Epsom, United Kingdom) using a T3000 Biometra thermocycler (Westburg, Leusden, The Netherlands) with the following thermal cycling profiles: a 15 minute initial incubation at 94 °C followed by 40 cycles of a 30 second dissociation phase at 94 °C, 30 seconds at 60 °C for the annealing process and 30 seconds at 72 °C for elongation, with a subsequent cooling down phase at 72 °C for five minutes. The PCR product obtained was 100 bp (base pairs) long.

Gel electrophoresis

Following PCR amplification of the sample DNA, 10 µl of the PCR product was mixed with loading buffer and placed into the available slots within a 2 % agarose gel (Eurogentec, Seraing, Belgium). A DNA ladder (37.5 ng/nl) was also placed into each gel at 5 µl volumes. The samples were then allowed to migrate at 100 Volts for thirty minutes before being placed in ethidium bromide (1 µg/ml stock solution) for 15 to 20 minutes. The primary PCR results were subsequently identified after visualisation under UV light.

Goat infection

Fresh camel blood (400 µl) was collected from the jugular vein of the camel and inoculated (i.p.) into several NMRI mice. Once a high parasitaemia had established itself within a mouse, blood was collected via cardiac puncture and mixed with phosphate buffered saline with glucose (PSG) to provide the trypanosome suspension used for experimental goat infection. The isolated strain was also cryopreserved by mixing the camel blood 1:1 with a freezing solution, containing 20 % glycerine in PBS, followed by storage at -80 °C.

Prior to experimental infection with *T. evansi*, a 4 ml blood sample from the jugular vein was taken from each goat and diagnostic methods were performed to ensure there was no previous evidence of *T. evansi* infection. Any goats demonstrating such evidence were removed from the project. Thereafter all goats were experimentally infected intravenously (i.v.), with 10^6 parasites in 1ml of a *T. evansi* strain, previously isolated from a dromedary camel in the south of Gran Canaria island.

Goat treatment

One month post-infection, a 4 ml blood sample was again taken from each goat via the jugular vein and diagnostic tests were carried out to establish that all goats were indeed infected with *T. evansi*. Following this, the goats were weighed and treatment was applied to the various groups of goats, according to the test compounds and treatment schedules depicted in *Table 6.1*. All test compounds were administered to the goats through an i.m. route and the goats were given four applications of their respective compound doses, 48 hours apart, on the following days: 32, 34, 36 and 38, post-infection. Thereafter, follow up diagnostic methods

were carried out for five months post treatment, to determine the curative efficacy of the compounds tested.

Table 6.1. The test compounds and treatment schedules used for the different groups of goats along with the number of goats in each group for the *T. evansi* efficacy trial.

Group	Number of goats	Test compound	Treatment schedule (mg/kg x 4)
1	4	None	None
2	3	Diminazene	5
3	3	DB 75	2.5
4	3	DB 867	2.5
5	3	DB 1192	2.5
6	3	DB 75	1.25
7	3	DB 867	1.25
8	3	DB 1192	1.25

Results

Table 6.2 demonstrates the results obtained, when goats were experimentally infected with *Trypanosoma evansi* and the parasitaemia was assessed using three different diagnostic methods. The results seen before initial infection was carried out, show that one goat (number 7328) was weakly positive (+) for the CATT / *T. evansi* test. Any goats revealing evidence of previous or current infections with *T. evansi* were not used in this experimental efficacy study and therefore this goat (number 7328) was removed from the project and no further tests were performed with it. However, all remaining goats tested negative for all three diagnostic methods used and were thus divided up into their allocated groups before initial infection was carried out.

Four days after infection, all goats were positive for the haematocrit centrifugation test, with motile trypanosomes seen in all blood samples taken from the goats. All the goats were similarly positive for PCR as would be expected, since a parasitaemia was present in all goats, thus parasite DNA was also present in all samples. Nevertheless, the CATT / *T. evansi* results were negative for all goats tested, since the sampling took place four days after the

goats had been experimentally infected, hence indicating that no antibodies could have developed in this short time to produce a positive antibody reaction.

Table 6.2. Status of each goat, before experimental infection with *T. evansi*, four days post infection and one month after infection and before compound treatment, involving the haematocrit centrifugation technique (HCT), the card agglutination test for trypanosomes (CATT) and the polymerase chain reaction method (PCR). The results are depicted as negative (-) or positive (+) for the HCT and PCR methods and strongly positive (+++), intermediate (++), weak (+) or negative (-) for the CATT test, along with goats, which died (*D*) or were removed from the project (*Rem*).

Group	Compound treatment	Goat No.	Before infection			Post infection			Before treatment		
			HCT	CATT	PCR	HCT	CATT	PCR	HCT	CATT	PCR
		7328	-	+	-	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
		7399	-	-	-	+	-	+	*D*	*D*	*D*
1	Control	2255	-	-	-	+	-	+	+	+++	+
		7397	-	-	-	+	-	+	+	+++	+
		7374	-	-	-	+	-	+	+	+++	+
		7314	-	-	-	+	-	+	+	+++	+
2	Diminazene	1773	-	-	-	+	-	+	+	+++	+
	5 mg/kg	7396	-	-	-	+	-	+	+	+++	+
		7388	-	-	-	+	-	+	+	+++	+
3	DB 75	7390	-	-	-	+	-	+	+	+++	+
	2.5 mg/kg	7324	-	-	-	+	-	+	+	+++	+
		5406	-	-	-	+	-	+	+	+++	+
4	DB 867	7319	-	-	-	+	-	+	+	+++	+
	2.5 mg/kg	5674	-	-	-	+	-	+	+	+++	+
		7386	-	-	-	+	-	+	+	+++	+
5	DB 1192	7379	-	-	-	+	-	+	+	+++	+
	2.5 mg/kg	7387	-	-	-	+	-	+	+	+++	+
		7361	-	-	-	+	-	+	+	+++	+
6	DB 75	5622	-	-	-	+	-	+	+	+++	+
	1.25 mg/kg	7366	-	-	-	+	-	+	+	+++	+
		7369	-	-	-	+	-	+	+	+++	+
7	DB 867	7372	-	-	-	+	-	+	+	+++	+
	1.25 mg/kg	7341	-	-	-	+	-	+	+	+++	+
		7339	-	-	-	+	-	+	+	+++	+
8	DB 1192	7384	-	-	-	+	-	+	+	+++	+
	1.25 mg/kg	7383	-	-	-	+	-	+	+	+++	+
		7360	-	-	-	+	-	+	+	+++	+

The sampling procedure performed before compound treatment was applied, was carried out on day 31 post-infection. Unfortunately, one goat (number 7399) showed severe clinical signs of *T. evansi* infection and died (day 26, post-infection) before treatment could be applied. No further tests could be performed with this goat and it was removed from the project. The remaining twenty-five goats on the other hand, produced strongly positive (+++) antibody reactions to the CATT / *T. evansi* test, at one month post-infection. Positive results were still seen with the haematocrit centrifugation technique and the PCR test demonstrating that a constant parasitaemia was still present.

After compound treatment, the goats were observed for a further five month follow up, using the same three diagnostic methods, to determine the curative efficacy of the three lead diamidine compounds, DB 75, DB 867 and DB 1192. The follow up results obtained, when using the haematocrit centrifugation technique (HCT), are shown in *Table 6.3*. A positive (+) result indicated that motile trypanosomes were seen within the buffy coat layer, after the blood samples had been centrifuged in the capillaries. A negative (-) result means that no motile trypanosomes were seen. The follow up part of the efficacy study lasted for 150 days (five months) and started 24 hours after the final compound application had been administered to those goats receiving treatment. Group 1 acted as the control group which received no compound treatment at all. All four goats within this group (numbers 2255, 7397, 7374 and 7314) demonstrated motile trypanosomes in each of their blood samples. Two goats (numbers 2255 and 7374) remained positive with a constant parasitaemia throughout the length of the follow up study (150 days). However, another two control goats (numbers 7314 and 7397) died during the study period on days 120 and 161 post-infection, respectively, due to severe clinical manifestation of *T. evansi* infection. Nevertheless, a constant parasitaemia was still observed in these two control goats.

All three goats allocated to Group 2 (numbers 1773, 7396 and 7388) were given four 5 mg/kg treatment applications of the positive control compound, diminazene. Thereafter, all three goats remained negative with the haematocrit centrifugation technique for the total length of the follow up study (150 days). A similar result was demonstrated by the compounds DB 75 and DB 867, both at the 2.5 mg/kg compound dose, where all goats in these two groups (Groups 3 and 4, respectively) produced negative findings for trypanosomes with the HCT. In Group 5, treatment with DB1192 at 2.5 mg/kg appeared insufficiently effective and motile trypanosomes were observed 2 weeks (14 days) after the final compound administration in two of the goats (numbers 7379 and 7387). These two goats were therefore removed from the project and no further tests were performed with them. The third goat

Table 6.3. A five month follow up of an efficacy study for a *T. evansi* experimentally infected goat model, using the haematocrit centrifugation technique (HCT), after treatment with Diminazene, DB 75, DB 867 or DB 1192 at various compound doses. The results are depicted as negative (-) or positive (+) for motile trypanosomes observed in goat blood samples, along with goats, which died (*D*) or were removed from the project (*Rem*) during the study period.

Group	Compound treatment	Goat No.	Follow up goat efficacy using haematocrit centrifugation technique (HCT) post treatment (in days)															
			1	7	14	21	28	35	42	49	56	70	84	98	112	126	140	150
1	Control	2255	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		7397	+	+	+	+	+	+	+	+	+	+	+	+	+	*D*	*D*	*D*
		7374	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		7314	+	+	+	+	+	+	+	+	+	+	*D*	*D*	*D*	*D*	*D*	*D*
2	Diminazene	1773	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5 mg/kg	7396	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		7388	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	DB 75	7390	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2.5 mg/kg	7324	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		5406	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	DB 867	7319	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2.5 mg/kg	5674	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		7386	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	DB 1192	7379	-	-	+	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
	2.5 mg/kg	7387	-	-	+	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
		7361	-	-	-	-	-	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*
6	DB 75	5622	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.25 mg/kg	7366	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		7369	-	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*
7	DB 867	7372	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	1.25 mg/kg	7341	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
		7339	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
8	DB 1192	7384	-	-	+	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
	1.25 mg/kg	7383	-	+	+	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
		7360	-	-	+	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*

(number 7361) was left within the project however, since motile trypanosomes were not detected within the blood samples of this goat. Nonetheless, this third goat (number 7361) died on day 34 post treatment.

Group 6 contained goats treated with DB 75, at half the dosage (1.25 mg/kg) used for Group 3 (2.5 mg/kg). One of the three goats (number 7369) was suffering from severe clinical symptoms of *T. evansi* infection before treatment even began, but was still included in the treatment schedule. Even so, the lead compound was not able to exert a beneficial effect quick enough and this goat died just 2 days post treatment. The two goats left in Group 6 (numbers 5622 and 7366) on the other hand, remained negative for the complete duration of the follow up study with the haematocrit centrifugation technique.

The goats in Group 7 were treated with DB 867 at half the dose (1.25 mg/kg) of that applied in Group 4 (2.5 mg/kg). All three goats in this group (numbers 7372, 7341 and 7339) remained negative for 149 days post treatment, when tested with the HCT. On the final day of the follow up study (day 150), all three goats presented motile trypanosomes in their blood.

In the final group (Group 8) treated with 1.25 mg/kg of DB 1192, motile trypanosomes were detected after only 7 days post treatment in one of the goats (number 7383). One week later (day 14), motile trypanosomes were seen in all three goats within Group 8 (numbers 7384, 7383 and 7360) and consequently, all three goats were removed from the project and no further tests were performed on them.

The five month follow up results, obtained for the card agglutination test for trypanosomes (CATT / *T. evansi*), are shown in *Table 6.4*. The results are based on the intensity of an agglutination reaction, occurring between goat plasma samples together with an antigenic reagent, to determine the antibody response. The positive results are illustrated as a strong antibody reaction (+++), an intermediate (++) or weak (+) response or a negative (-) result, where no antibody reaction was observed.

For the untreated control goats (Group 1), where a constant parasitaemia was established, all four goats (numbers 2255, 7397, 7374 and 7314) remained strongly positive with the CATT / *T. evansi* test for the duration of the follow up study. With the diminazene (5 mg/kg) positive control goats (Group 2), a decreased intensity of antibody reactions throughout the follow up study were detected for all three goats (numbers 1773, 7396 and 7388). In addition, two goats (numbers 7388 and 1773) produced negative CATT / *T. evansi* responses, 7 days and 56 days respectively, post treatment.

In Groups 3 and 4, a similar trend was demonstrated and the CATT / *T. evansi* test results decreased in intensity from strongly positive to weakly positive. In the case of DB 75

Table 6.4. A five month follow up of an efficacy study for a *T. evansi* experimentally infected goat model, using a card agglutination test (CATT / *T. evansi*), after treatment with Diminazene, DB 75, DB 867 or DB 1192 at various compound doses. Results are depicted as strongly positive (+++), intermediate (++), weak (+) or negative (-) for agglutination, when goat plasma was mixed with an antigenic reagent, along with goats, which died (*D*) or were removed from the project (*Rem*) during the study period.

Group	Compound treatment	Goat No.	Follow up goat efficacy using a card agglutination test (CATT/*T. evansi*) post treatment (in days)															
			1	7	14	21	28	35	42	49	56	70	84	98	112	126	140	150
1	Control	2255	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		7397	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	*D*	*D*	*D*
		7374	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		7314	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	*D*	*D*	*D*	*D*	*D*	*D*
2	Diminazene	1773	+++	+++	++	++	++	+	+	+	-	-	-	-	-	-	-	-
	5 mg/kg	7396	+++	+++	+++	+++	+++	+++	++	++	++	++	++	++	++	++	++	++
		7388	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	DB 75	7390	+++	+++	+++	+++	+++	++	++	++	+	+	+	+	+	+	+	+
	2.5 mg/kg	7324	+++	+++	+++	+++	+++	++	+	+	-	-	-	-	-	-	-	-
		5406	+++	+++	+++	+++	+++	++	++	++	++	++	+	+	+	+	+	+
4	DB 867	7319	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2.5 mg/kg	5674	+++	+++	++	+	+	+	+	+	+	+	-	-	-	-	-	-
		7386	+++	+++	+++	++	++	+	+	+	+	+	+	+	+	+	+	+
5	DB 1192	7379	+++	+++	+++	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
	2.5 mg/kg	7387	+++	+++	+++	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
		7361	+++	++	++	+++	+++	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*
6	DB 75	5622	+++	++	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	1.25 mg/kg	7366	+++	+++	+++	+++	+++	++	++	++	++	++	++	++	++	++	++	++
		7369	+++	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*
7	DB 867	7372	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++	+++
	1.25 mg/kg	7341	+++	++	++	++	++	++	++	++	+	+	+	+	+	+	++	+++
		7339	+++	++	+	+	+	+	+	+	+	+	+	+	+	+	++	+++
8	DB 1192	7384	+++	+++	+++	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
	1.25 mg/kg	7383	+++	+++	+++	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
		7360	+++	+++	+++	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*

at 2.5 mg/kg, one goat (number 7324) became negative on day 56 post treatment, as did two goats from DB 867 at 2.5 mg/kg (numbers 7319 and 5674), becoming negative on days 14 and 84, respectively, post treatment. Group 5 goats (numbers 7379, 7387 and 7361), treated with DB 1192 at 2.5 mg/kg, expressed strong to intermediate positive antibody reactions and were removed from the project based on microscopic detection of the parasite. Equally so for the lower dose of DB 1192 of 1.25 mg/kg (Group 8), where all three goats (numbers 7384, 7383 and 7360) displayed strong positive reactions and were subsequently removed from the project after two weeks follow up.

For the goat suffering from severe clinical infection in Group 6 (DB 75 at 1.25 mg/kg), a strong positive reaction was noted, before this goat died (number 7369) on day 2 post treatment. The remaining two goats in Group 6 showed a negative reaction from day 56 post treatment onwards (number 5622) and an intermediate response from day 35 onwards (number 7366), respectively. Interestingly, the three goats in Group 7 (numbers 7372, 7341 and 7339), treated with DB 867 at a 1.25 mg/kg dose, revealed primarily a decrease from strongly positive to intermediate (and even to weak in goat numbers, 7341 and 7339), until approximately day 126 post treatment, after which an increase in antibody reaction (back to strongly positive) was subsequently detected.

Table 6.5 shows the data obtained, when the PCR method was used for the five month efficacy follow up study. A positive reaction (+) for the PCR method confirmed the presence of parasite DNA within the goats' blood, whereas a negative result (-) indicated that no parasite DNA was found in the goats' blood at that point. As would be expected, all four untreated control goats (numbers 2255, 7397, 7374 and 7314) within Group 1, demonstrated a constant parasitaemia and hence positive PCR results for the duration of the follow up study. In comparison, Groups 2, 3 and 4, representing the positive control group, treated with diminazene (goat numbers 1773, 7396 and 7388), the 2.5 mg/kg dose group of DB 75 (goat numbers 7390, 7324 and 5406) and the 2.5 mg/kg dose group of DB 867 (goat numbers 7319, 5674 and 7386), respectively, all produced negative PCR results.

For the three goats in Group 5 (numbers 7379, 7387 and 7361), treated with 2.5 mg/kg of DB 1192, a positive PCR result was detected on days 7, 14 and 28 post treatment, respectively. Two of the goats within Group 5 (numbers 7379 and 7387) were removed on day 14 post treatment (based on microscopic detection of the parasite in the blood) and no further tests were performed on them. The third goat (number 7361), having died on day 34 post treatment, had previously shown a positive result with the PCR method on day 28 post treatment, even though no parasites were seen in the blood at this time with the HCT method.

Table 6.5. A five month follow up of an efficacy study for a *T. evansi* experimentally infected goat model, using the polymerase chain reaction (PCR), after treatment with Diminazene, DB 75, DB 867 or DB 1192 at various compound doses. The results are depicted as negative (-) or positive (+) for the presence of parasite DNA in goat blood samples, along with goats, which died (*D*) or were removed from the project (*Rem*) during the study period.

Group	Compound treatment	Goat No.	Follow up goat efficacy using polymerase chain reaction (PCR) post treatment (in days)															
			1	7	14	21	28	35	42	49	56	70	84	98	112	126	140	150
1	Control	2255	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		7397	+	+	+	+	+	+	+	+	+	+	+	+	+	*D*	*D*	*D*
		7374	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		7314	+	+	+	+	+	+	+	+	+	+	*D*	*D*	*D*	*D*	*D*	*D*
2	Diminazene	1773	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5 mg/kg	7396	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		7388	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	DB 75	7390	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2.5 mg/kg	7324	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		5406	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	DB 867	7319	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2.5 mg/kg	5674	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		7386	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	DB 1192	7379	-	+	+	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
	2.5 mg/kg	7387	-	-	+	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
		7361	-	-	-	-	+	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*
6	DB 75	5622	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1.25 mg/kg	7366	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		7369	+	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*D*
7	DB 867	7372	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
	1.25 mg/kg	7341	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
		7339	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
8	DB 1192	7384	+	+	+	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
	1.25 mg/kg	7383	+	+	+	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*
		7360	+	+	+	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*	*Rem*

Likewise, the severely affected goat (number 7369) in the lower dose group of DB 75 (Group 6), presented a positive result for parasite DNA present in the blood, one day after treatment, before it eventually died and was removed from the project. The remaining two goats (numbers 5622 and 7366) however, were PCR negative for the rest of the follow up study (150 days post treatment).

Unfortunately, the three goats (numbers 7372, 7341 and 7339) in Group 7 (DB 867 treatment at 1.25 mg/kg), having shown negative PCR results until day 125 post treatment, relapsed and parasitic DNA was detected in all three goats on days 126 (for goat numbers, 7372 and 7341) and 140 (for goat number, 7339) post treatment. They were not removed initially from the project, since motile trypanosomes were only observed on day 150, post treatment. All goats within Group 8 (goat numbers, 7383, 7384 and 7360), treated with 1.25 mg/kg of the compound, DB 1192, never became PCR negative after compound treatment, thus demonstrating complete inefficacy at this dose.

Discussion

The aim of this study was to primarily establish a higher animal model using goats, which could be infected with *Trypanosoma evansi*. Thereafter, three selected lead diamidine compounds (DB 75, DB 867 and DB 1192) were to be investigated for their curative efficacy at several doses, in comparison with the standard compound, diminazene aceturate.

Previous attempts to infect goats with *T. evansi* has lead to various problems, as seen in a goat study conducted in the Philippines using a local parasite strain (*Dargantes et al., 2005*), where several goats developed an acute infection, the virulence of which ended in fatal consequences for the animals. Goat studies performed in the Canary Islands (*Gutierrez et al., 2004*a; *Gutierrez et al., 2004*b; *Morales et al., 2006*) reported the onset of sub-clinical symptoms, when infection advances to the chronic form. However, severe clinical infection and specific pathological features have also been described in a study on small east African goats (*Ngeranwa et al., 1993*). Coupled with the limited availability of good diagnostic tests and a low or even intermittent parasitaemia, accurate detection of this parasite is complicated further. It was anticipated that, by using a less virulent parasite strain (endemic in the Canary Islands) at a higher parasite concentration (10^6/ml), a steady and constant parasitaemia could be achieved to ease the dilemma of accurate parasite detection.

Furthermore, a range of diagnostic methods were applied to measure the presence of *T. evansi* in the goats, using several approaches. A parasitological test (HCT) was applied to provide direct demonstration of the parasite itself, an approach that relies mainly on the existence of a regular parasitaemia. The standard serological test (CATT / *T. evansi*) was used to measure the level of antibodies during the course of the goat study. It is debatable as to whether an organism is considered completely cured, when antibody tests remain positive. Since most serological techniques are unable to differentiate between recent and past infections, the decision to use the CATT / *T. evansi* test in this study was essentially as a guide to disease progression, especially for understanding the five month follow up part of this investigation. The final diagnostic method used was a molecular based technique (PCR), which aims to detect the presence of parasitic DNA, even when a parasitaemia is too low to be detected by microscopy or when serological results are indecisive. Due to the specificity and sensitivity of such molecular based techniques, they tend to become the gold standard methods, which was also the case in this study. However, this PCR technique performed in this efficacy trial was used for the first time for diagnostic purposes. Nevertheless, the relationship seen between the PCR and the HCT method in this pilot study is considered to be accurate and well correlated.

The resulting positive CATT / *T. evansi* result given by one goat (number 7328) at the start of the study, implied that this goat may have been previously exposed to *T. evansi* infection and was consequently removed from the project. After experimental infection, two goats (numbers 7399 and 7369) demonstrated severe clinical symptoms of infection and died subsequently. From the control group of goats, which received no compound treatment, a chronic, sub-clinical progression of *T. evansi* infection was observed. However, two control goats (numbers 7314 and 7397) died during the follow up study, demonstrating the fact that treatment is critical for the survival of infected animals.

Complete curative efficacy was seen for the whole five month follow up trial in the positive control group, treated with a 5 mg/kg dose of diminazene. Additional complete curative efficacy was demonstrated in the goats treated with both 2.5 mg/kg and 1.25 mg/kg doses of the compound, DB 75. It may even be possible that this compound could effectively cure *T. evansi* infected goats at a lower dose than 1.25 mg/kg. However, another goat efficacy trial would need to be performed to confirm whether 1.25 mg/kg really is the lowest curative dose or not. With the compound DB 867, curative efficacy was seen in the higher treatment dose (2.5 mg/kg) for the entire five month follow up study and appeared similarly so for the lower (1.25 mg/kg) compound dose for the first four months. However, on day 140, all goats

treated with DB 867 at 1.25 mg/kg demonstrated positive PCR results, yet parasites were only visually detected in the blood after 150 days, post treatment. An interesting result was observed in the serological findings of these goats, which did not produce negative CATT / *T. evansi* results contrary to some of the goats treated with the higher dose (2.5 mg/kg). Instead an increased response in antibody level was noted from days 140 (post treatment) onwards. Since the goats were housed in fly-proof facilities, the potential risk of re-infection (experimental or natural) in this case is small, together with the knowledge that the control goats (which underwent no compound treatment) were also housed in a separate fly-proof pen within the research building. The exact reasons for understanding why this compound caused a relapse at this stage of the efficacy study may be made clearer after a pharmacokinetic investigation into DB 867 has been performed.

The third lead diamidine compound tested in this efficacy trial (DB 1192) was not effective at either dose tested. Goats treated with 2.5 mg/kg of DB 1192 presented trypanosomes within the blood, 14 days after treatment was given. The antibody levels of these goats remained high (++ to +++) and DNA was detected earlier, between one and two weeks, post treatment. One goat (number 7361) remained PCR negative for just less than one month, yet this compound was still insufficient to provide comparable curative efficacy to that seen with the standard compound, diminazene. A treatment failure was also shown in the lower dose group (1.25 mg/kg) of DB 1192, where none of the goats ever became PCR negative and the serological results remained strongly positive (+++).

In conclusion, this pilot efficacy trial into *T. evansi* infected goats has enabled three selected lead diamidine compounds to be evaluated, of which two (DB 75 and DB 867) demonstrated better efficacy than the standard compound (diminazene aceturate). The compound DB 75 was more effective at a quarter of the dose used (1.25 mg/kg), than diminazene and DB 867 was more effective at half the dose used (2.5 mg/kg). Therefore, two lead diamidine compounds (DB 75 and DB 867) have successfully been identified through this pilot "proof of concept" trial, as potential clinical candidates against *T. evansi* infection.

Acknowledgments

The authors wish to acknowledge the hard work and effort invested by the staff of the Hospital Clinicó Veterinario in Arucas, Gran Canaria and for the kind use of their facilities.

Furthermore, the additional work and assistance of Titos Cau and Stijn Rogé, at the Institute of Tropical Medicine in Antwerp, Belgium, is greatly appreciated with regard to the evaluation and results from the PCR technique.

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Pharmacokinetic analysis of DB 75, DB 867 and DB 1192 in *Trypanosoma evansi* infected goats

Kirsten Gillingwater[1], Carlos Gutierrez[2], Arlene Bridges[3], Huali Wu[4] and Reto Brun[1]

[1]Parasite Chemotherapy, Swiss Tropical Institute, Socinstrasse 57, 4002 Basel, Switzerland
[2]Veterinary Faculty of the University of Las Palmas, 3416 Arucas, Canary Islands, Spain
[3]School of Pharmacy, University of North Carolina, Chapel Hill NC, United States of America
[4]Department of Pathology, University of North Carolina, Chapel Hill NC, United States of America

Abstract

Pharmacokinetic analysis is important to create successful drug therapy and optimal treatment regimens, to enable the drug concentration within the body to remain within the therapeutic window, but at the same time prevent adverse or toxic effects. Three lead diamidine compounds were selected based on their previous activity *in vitro* and *in vivo*, as well as on preliminary toxicity results obtained from healthy goats. The curative efficacy of these lead diamidine compounds (DB 75, DB 867 and DB 1192) was further tested in an experimentally infected *Trypanosoma evansi* goat model and subsequently a pharmacokinetic study was carried out. Blood samples were collected at the following time points of 0.5, 1, 2, 4, 8, 24, 48, 96 and 192 hours, after the intramuscular administration of the last compound injection. The plasma samples produced were analysed using an HPLC/MS-MS technique and pharmacokinetic profiles and parameters were determined.

The pharmacokinetic results obtained in this study depict clearly the reasoning behind the compound treatment failures seen for DB 1192, when used in an experimentally infected *T. evansi* goat model. The low compound concentrations in goat plasma of DB 1192 fall far below the minimal compound concentrations required to reach therapeutic levels, whereas plasma concentrations for diminazene (standard compound), DB 75 and DB 867 remain well within therapeutic concentrations. Hence, curative efficacy is provided by diminazene at 5 mg/kg, by DB 75 at 2.5 mg/kg and 1.25 mg/kg and by DB 867 at 2.5 mg/kg. Although the concentrations of DB 867 at 1.25 mg/kg were within the therapeutic window, other physiological factors may be responsible for the relapse seen at 4.5 months post treatment with this compound.

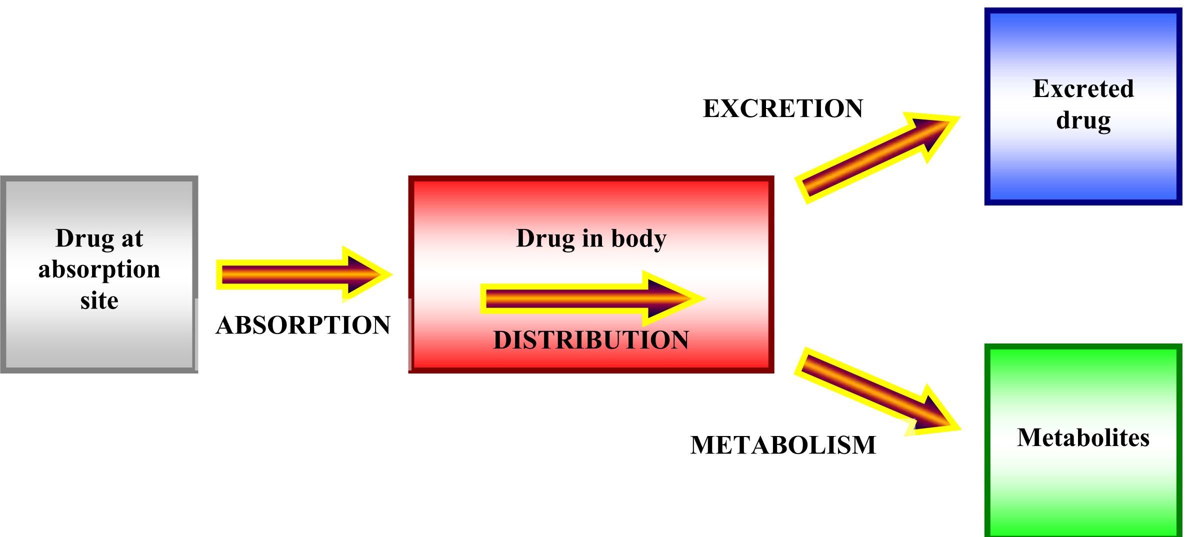
Keywords: *Trypanosoma evansi*, pharmacokinetics, diamidines, goat model, chemotherapy.

Introduction

Pharmacokinetics is the quantitation of the time course of a drug and its metabolites in the body and the development of appropriate models to describe the observations. It can be applied in order to measure and understand the kinetics of drugs in the body, thus providing valuable data whilst following the processes involved after drug administration. *Figure 7.1*

shows a simple pharmacokinetic model, which diagrammatically depicts the different processes a drug goes through after it has been administered. Such pharmacokinetic information is required to carry out successful drug therapy and avoid the occurrence of adverse or toxic effects.

Figure 7.1. Pharmacokinetic model to display the processes encountered after a drug is administered and how it moves to various compartments (boxes) via the processes of absorption, distribution, metabolism and excretion (shown by the arrows).



Drugs can be administered at different sites, which can be grouped as intravascular (directly into the blood, via an intra-venous or intra-arterial route) or extravascular, where the drug needs to be absorbed before it can enter the blood (via oral, sublingual, buccal, intramuscular, subcutaneous, dermal, pulmonary or rectal routes). Absorption of a drug is the process by which the unchanged drug proceeds from the administration site towards the measurement site within the body. However, drug can be lost at the site of administration before it reaches the measurement site, which would contribute to a decrease in the perceived absorption of that drug. This does not apply to intravascular routes of application, since the drug does not need to be absorbed. Nevertheless, monitoring the amount of drug in blood or plasma enables the assessment of entry of a drug into the body. Once a drug is in the blood, it is distributed (reversibly) to various tissues and organs around the body. The rate and extent of distribution is dependent on how well each tissue is supplied with blood, the size of the tissue, the binding of the drug to plasma proteins and DNA and the permeability of tissue membranes. If a drug leaves the measurement site and does not return, then it has undergone elimination and not distribution. Elimination (usually carried out by organs such as the liver

and kidneys) is the irreversible loss of drug from the measurement site and can occur via two processes: excretion and metabolism. Excretion can be defined as the irreversible loss of the chemically unaltered drug (usually performed by the kidneys) and metabolism is the conversion of one chemical species into another to create metabolites (usually performed by the liver). Together distribution and elimination are the components of disposition (*Rowland and Tozer, 1989*).

Three lead diamidine compounds were selected for outstanding *in vitro* and *in vivo* efficacy against the animal pathogenic protozoan parasite, *Trypanosoma evansi*. The curative efficacy of these three compounds, DB 75, DB 867 and DB 1192, were assessed in an experimentally infected *T. evans*i goat model. A pharmacokinetic study was additionally carried out with the aim of providing preliminary pharmacokinetic profiles for the three compounds and to determine several pharmacokinetic parameters to establish a clearer understanding of the processes occurring after these compounds were administered to infected goats. An intramuscular route of application was chosen based on the practicalities and safety aspects of diamidine compounds given through this type of application. It was previously seen that intramuscular injections of DB 75, DB 867 and DB 1192 caused no acute signs of toxicity in healthy goats at a maximum compound dose of 4 mg/kg. The kinetics which occurs after compounds are given intramuscularly, rather than through an intravenous route directly into the blood, differ in several ways and must be taken into account, when analysing the results.

Materials and methods

Trypanosoma evansi strain

A *Trypanosoma evansi* strain, designated as the Canaries (Rubio) strain, was originally isolated by K Gillingwater in 2006, from a twenty-two year old dromedary camel, in a farm on Gran Canaria, belonging to the Canary Islands, Spain. The camel blood was immediately inoculated into mice and after a single mouse passage, was then used for experimental infection of the goats.

Goats

This study was approved by the ethical committee of the Hospital Clinicó Veterinario, Gran Canaria. Twenty-seven female Canarian goats, weighing between 20 - 52 kg and older than 6 months, were purchased from a local dairy farmer. After transportation to the veterinary research facility, the goats were placed inside open pens and allowed to acclimatise for a week to their new surroundings. Thereafter, the goats were given numbered ear tags, weighed and checked for current or previous *T. evansi* infection. Any goats showing positive results for previous *T. evansi* infection were removed from the project. The remaining goats were then divided into test groups, given oxytetracycline against nasal exudate and ivermectin as a standard anti-helminthic, before being placed into fly-proof pens. Throughout the study period, the goats were given food (pelleted nutrients, dried corn and hay), three times a day and water was provided *ad libitum*.

Standard trypanocidal compound

Diminazene aceturate (D-7770, Sigma, St. Louis, MO, USA) was used as the standard control compound in this pharmacokinetic goat study. Berenil®, the officially registered veterinary drug, contains only 52.5 % of the active component, diminazene aceturate.

Lead diamidines

The diamidine compounds were originally designed and synthesised by the chemist, David Boykin. The compounds DB 75, DB 867 and DB 1192 were used as the test compounds in this pharmacokinetic goat study, based on their previous *in vitro* and *in vivo* activity and preliminary toxicity within a mouse model, together with the initial toxicity results obtained from a goat toxicity trial. The data for these previous investigations can be viewed in chapters 3, 4 and 5, respectively.

Stock solutions

A 900 mg amount of the compounds, DB 75, DB 867 and DB 1192 were weighed out in powder form and dissolved in 36 ml of sterile distilled water with 10 % DMSO, to provide a 25 mg/ml stock solution. In addition, a 450 mg amount of the compounds, DB75, DB 867

and DB 1192 were weighed out in powder form and dissolved in 36 ml of sterile distilled water with 10 % DMSO, to provide a 12.5 mg/ml stock solution. A 1800 mg amount of the standard compound, diminazene aceturate was weighed out in powder form and dissolved in 36 ml of sterile distilled water with 10 % DMSO, to provide a 50 mg/ml stock solution. These stock solutions were then applied in the pharmacokinetic goat study as 10 kg /ml chemotherapeutic solutions and were made fresh on the day of the first administration. Thereafter, the stock solutions were kept stored at 4 °C.

Experimental infection of goats

Prior to experimental infection with *T. evansi*, a 4 ml blood sample from the jugular vein was taken from each goat and diagnostic methods were performed to ensure there was no previous evidence of *T. evansi* infection. Any goats demonstrating such evidence were removed from the project. Thereafter all goats were experimentally infected intravenously (i.v.), with 10^6 parasites in 1 ml of a *T. evansi* strain, previously isolated from a dromedary camel in the south of Gran Canaria island.

Goat treatment

One month post-infection, a 4 ml blood sample was again taken from each goat via the jugular vein and diagnostic methods were carried out to establish that all goats were indeed infected with *T. evansi*. Following this, the goats were weighed and treatment was applied to the various groups of goats, according to the compounds and treatment schedules depicted in *Table 7.1*. All test compounds were administered to the goats through an i.m. route and the goats were given four applications of their respective compound doses, 48 hours apart, on the following days: 32, 34, 36 and 38, post-infection.

Blood samples (4 ml volumes) were taken for pharmacokinetic analysis from the goats at the following time points of 0.50, 1, 2, 4, 8, 24, 48, 96 and 192 hours after the administration of the fourth (and final) compound injection. In addition, blood samples were taken from all the goats, shortly before each compound injection was applied, to determine the trough levels.

Table 7.1. The test compounds and treatment schedules used for the different groups of goats along with the number of goats in each group for the pharmacokinetic study.

Group	Number of goats	Test compound	Treatment schedule (mg/kg x 4)
1	4	None	None
2	3	Diminazene	5
3	3	DB 75	2.5
4	3	DB 867	2.5
5	3	DB 1192	2.5
6	3	DB 75	1.25
7	3	DB 867	1.25
8	3	DB 1192	1.25

Collection of blood samples

Blood samples were collected using clean, sterile Vacutainer® (Becton Dickinson, Plymouth, UK) tubes of 4 ml volume, containing the anticoagulant, ethylene diamine tetra acetic acid (EDTA). The tubes were inverted several times, immediately after blood was taken from the jugular veins of the goats, to ensure equal distribution of the anticoagulant throughout the blood sample taken. The tubes were then kept on ice until they reached the laboratory for plasma preparation.

All blood samples taken at various time points, for each goat and each test compound, were centrifuged at 3000 *g* for ten minutes. Subsequently, the plasma was removed, placed into sterile polypropylene cryotubes and stored away from light at -80 °C.

Preparation of plasma samples

Plasma aliquots (25 µl volumes) were vortexed with 200 µl of a 12.5 nM internal standard (DB 103) dissolved in methanolic HCl (8:1 methanol: 1N HCl). Protein was precipitated by centrifugation (Beckman GS-6R) at 3000 rpm for 7 minutes at room temperature. The supernatant (120 µl) was then transferred to 96-well plates and evaporated under heated nitrogen (EVX Evaporator, Apricot Designs, USA). Thereafter, the samples were reconstituted in the plate, with 60 µl of high pressure liquid chromatography (HPLC) grade water (Fisher Scientific, USA), for analysis by mass spectrometry.

Calibrators were prepared separately for each analyte. Stock solutions of diminazene, DB 75, DB 867 and DB1192 were prepared in water. Aqueous dilutions of each stock solution were prepared to provide the following spiking solutions: 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250 and 500 μM. To produce the intended calibration solutions of 5, 10, 25, 50, 100, 250, 500, 1000, 2500 and 5000 nM, 0.6 μl of aqueous spiking solution was added to 60 μl of untreated commercial goat plasma. These calibrators were then treated in the same way as the plasma samples. Curves were then prepared and analysed in duplicate, at both the beginning and the end of the analytical run.

Mass spectrometry analysis

Concentrations of diminazene, DB 75, DB 867 and DB 1192 were determined by Liquid Chromatography/Triple Quadrupole Mass Spectrometry (HPLC/MS-MS). The HPLC/MS-MS system consisted of two solvent delivery pumps (Shimadzu Scientific, Colombia, MD, USA), a switching valve (Valco, Houston, TX, USA), a thermostated (6 °C) LEAP HTC auto-sampler (Carrboro, NC, USA) and an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA). Reversed-phase gradient chromatography was used to elute the compounds from an analytical column (Aquasil, C18 5 μm, 50 × 2.1 mm) at a flow rate of 0.75 ml / minute, following an 8 μl injection. Starting conditions for each injection included 95 % water with 0.1 % formic acid (v/v) and 5 % methanol with 0.7 % formic acid (v/v). This was held constant for 1 minute while the column eluted to waste. After 1 minute, the eluent was directed to the mass spectrometer and the organic phase (methanol with 0.1 % formic acid, v/v) increased linearly, reaching 90 % organic, 3.25 minutes post-injection. This was held for 0.5 minutes to wash the column. The column was then re-equilibrated to starting conditions for the final 1 minute. The total run time was 4.75 minutes.

The mass spectrometer was connected to the HPLC system by a TurboIon Spray® interface (MDS Sciex, San Francisco, CA, USA). Nitrogen, from a nitrogen generator (Peak Scientific, Bedford, MA, USA), was used as the curtain, nebulizer and collision gases. User controlled voltages, gas pressures and source temperature were optimised via direct infusion, for DB 103 (the internal standard), diminazene, DB 75, DB 867 and DB 1192. All compounds were analysed in positive ion mode, using the following transitions preset in multiple reaction monitoring scans: DB 103 (internal standard) - 385.2 to 271.2, diminazene - 282.2 to 254.1, DB 75 - 305.2 to 288.2, DB 867 - 306.3 to 289.2 and DB 1192 - 292.3 to 275.1. The

calibration curves were generated based on peak area ratios (analyte: internal standard) from 5 to 5000 nM. Curves for all analytes followed a linear y = mx + b regression with a $1/x^2$ weighting. Typical r values were 0.997 or greater and accuracy was between 80 and 120 %. Automated sample acquisition and data analysis was performed using Analyst software (version 1.4.1, Applied Biosystems, Foster City, CA, USA).

Pharmacokinetic parameters

The pharmacokinetic parameters were determined using Microsoft® Excel software, with a functional set of add-ins applied, together with a graphical programme (GraphPad Prism 5, GraphPad Software Inc, USA). Pharmacokinetic parameters measured and calculated included the peak concentration (C_{max}), time at which peak concentration occurs (T_{max}), the area under the curve (AUC), the area under the first moment curve (AUMC), the mean residence time (MRT), the half life ($t_{1/2}$) and the rate of elimination constant (K_{elim}). The MRT was calculated with the function, AUMC/AUC and the rate of elimination constant was obtained with the function, 1/MRT.

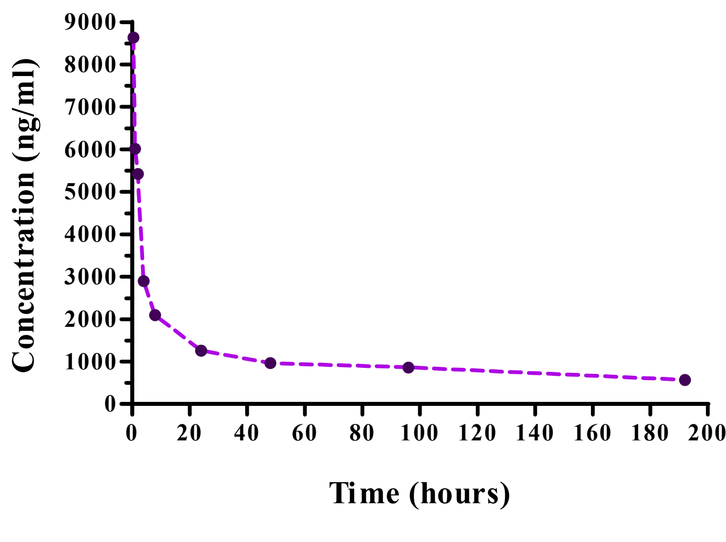
Results

The pharmacokinetic profiles and results for the pharmacokinetic analysis can be seen in *Figure 7.2* for the standard compound diminazene, *Figure 7.3* for the compound DB 75, *Figure 7.4* for the compound DB 867 and *Figure 7.5* for the compound DB 1192. The plasma samples taken were analysed by HPLC/MS-MS to determine the concentrations (in ng/ml) of each compound tested at each specific time point. From these values, the averages from the experimental goats in each group were calculated and concentration versus time curves were plotted with a line of best fit applied, corresponding to either the high or low compound dose tested.

In addition, the pharmacokinetic parameters calculated can be viewed in *Table 7.2* for *Trypanosoma evansi* experimentally infected goats, given four treatment doses of 2.5 mg/kg and in *Table 7.3* for *T. evansi* experimentally infected goats, given four treatment doses of 1.25 mg/kg. In both tables, the pharmacokinetic parameters for the standard compound,

diminazene, given to *T. evansi* experimentally infected goats at four treatment doses of 5 mg/kg, has also been included as a comparison.

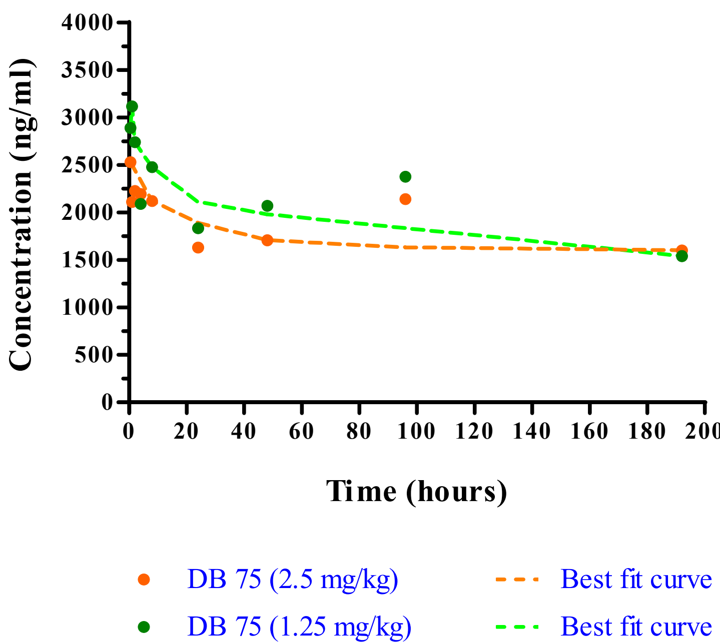
Figure 7.2. Pharmacokinetic profile obtained from the average compound concentrations in plasma samples, taken from all three goats in Group 2 for diminazene, when treated with four applications of 5 mg/kg, against time.



The peak concentration (C_{max}) for goats treated with diminazene, with four applications of 5 mg/kg doses, reached ≥ 8637 ng/ml after just ≤ 0.5 hours (T_{max}) after the fourth (and final) compound injection was given. The area under the curve was 193'589, whilst the area under the first moment curve was calculated as 13'683'170. The mean residence time (MRT) for diminazene was 70.7 hours, with an elimination rate constant (K_{elim}) of 0.01 hrs^{-1}. With this pharmacokinetic profile, the half life of diminazene was determined to be 59.1 hours.

The peak concentration produced for goats treated with the compound DB 75, with four applications of 2.5 mg/kg, was lower (≥ 2530 ng/ml) than the C_{max} produced by goats treated with four applications of DB 75 at 1.25 mg/kg (≥ 3115 ng/ml). However, it took longer for the lower dose (1.25 mg/kg) goats to reach this higher peak with a T_{max} of ≤ 1.0 hour, as compared to the T_{max} of the 2.5 mg/kg treated goats (≤ 0.5 hours). In addition, the peak concentration of goats treated with DB 75 at both doses was approximately three times lower than that seen with diminazene.

Figure 7.3. Pharmacokinetic profiles obtained from the average compound concentrations in plasma samples, taken from all three goats in Group 3 and both goats in Group 6 for DB 75, when treated with four applications of 2.5 mg/kg or four applications of 1.25 mg/kg, respectively, against time.

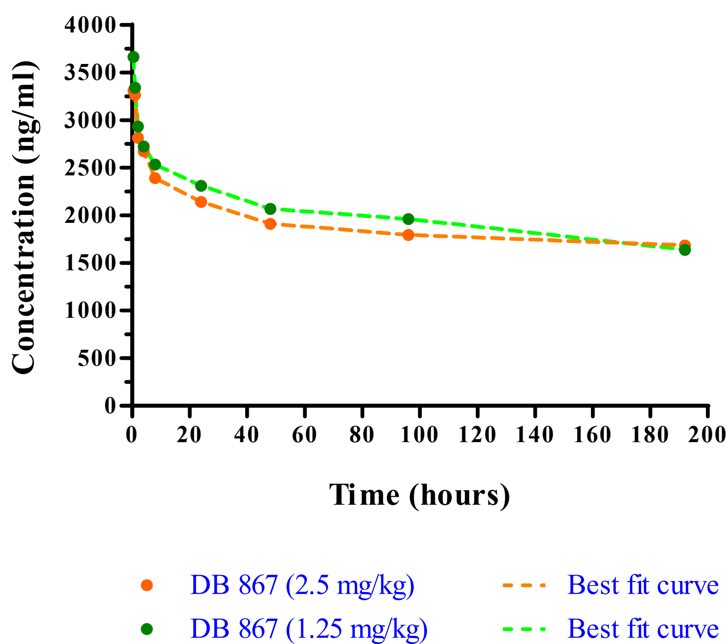


The AUC for both groups of goats treated with DB 75 were similar to one another (358’215 and 394’305, respectively), yet twice as large as the AUC calculated for diminazene. Again, the AUMC for both groups of DB 75 goats were similar to one another (33’469’105 and 35’295’790, respectively), but greater than the AUMC of diminazene by a factor of 2.5. The MRT for the 2.5 mg/kg group was 93.4 hours and the MRT for the 1.25 mg/kg group was 89.5 hours. The rate of elimination constant was equal for the DB 75 high and low dose treated goats and to that of diminazene (0.01 hrs^{-1}). The half life of the 1.25 mg/kg treated goats was determined to be 279.3 hours, whilst the half life of the 2.5 mg/kg treated goats was approximately 1.7 times longer at 473.7 hours. This is approximately five and eight times longer than the half life of diminazene.

The pharmacokinetic parameters measured for the goats treated with DB 867 at 2.5 mg/kg and 1.25 mg/kg doses show similar results to the pharmacokinetic parameters determined for DB 75, at the same 2.5 mg/kg and 1.25 mg/kg doses. The peak concentration achieved was higher in the 1.25 mg/kg group (≥ 3663 ng/ml), than in the 2.5 mg/kg group for DB 867 (≥ 3310 ng/ml). The time at which peak concentration occurred for both treatment schedules of DB 867 was the same (≤ 0.5 hours). The AUC and AUMC were similar, yet slightly larger in the 1.25 mg/kg group. The mean residence time for both groups was also

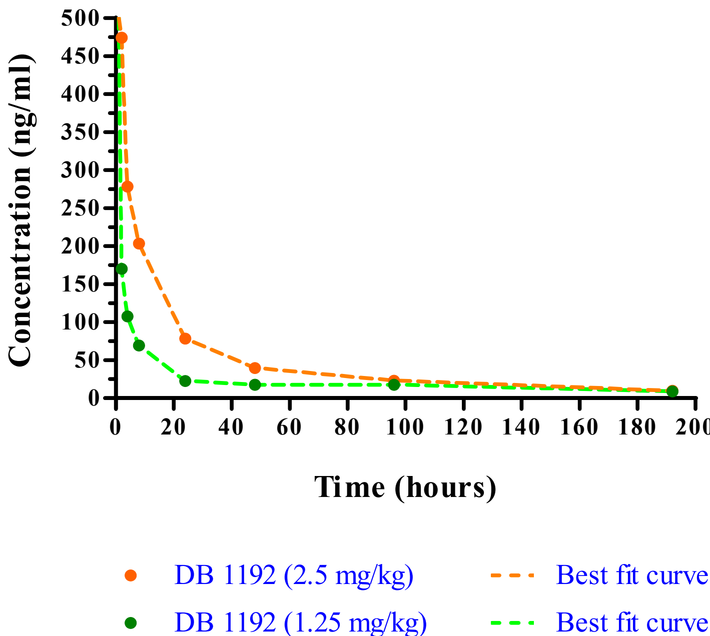
comparable (90.0 and 87.9 hours) and slightly longer than that of diminazene. The elimination rate constant was 0.01 hrs^{-1} for both DB 867 treatment groups and identical to the elimination rate constants for DB 75 treatment groups and diminazene. The half lives of DB 867 for the 2.5 mg/kg and 1.25 mg/kg doses were similar (221.4 hours and 202.5 hours, respectively) and were approximately four times greater than that of diminazene.

Figure 7.4. Pharmacokinetic profiles obtained from the average compound concentrations in plasma samples, taken from all three goats in Group 4 and from all three goats in Group 7 for DB 867, when treated with four applications of 2.5 mg/kg or four applications of 1.25 mg/kg, respectively, against time.



The C_{max} for DB 1192 was much lower for the 2.5 mg/kg (≥ 1563 ng/ml) and 1.25 mg/kg (≥ 836 ng/ml) treated goats compared to the peak concentrations of DB 75 and DB 867 compounds. Nevertheless, T_{max} remained the same at ≤ 0.5 hours. The AUC and AUMC for DB 1192 at 2.5 mg/kg (9'861 and 378'645) were smaller than the AUC and AUMC for DB 1192 at 1.25 mg/kg (4'809 and 251'434) and immensely small compared to the AUC and AUMC values of DB 75, DB 867 and diminazene. The MRT was longer in the lower dosed DB 1192 treatment schedule (52.3 hours) than in the higher dosed group (38.4 hours). The elimination rate constants were 0.03 hrs^{-1} and 0.02 hrs^{-1} for the 2.5 mg/kg and 1.25 mg/kg groups, respectively, making them also two times and three times greater than the elimination rate constants seen in DB 75, DB 867 and diminazene. The half lives of DB 1192 were determined to be around 30.5 hours and 37.0 hours for the high and low dosed groups.

Figure 7.5. Pharmacokinetic profiles obtained from the average compound concentrations in plasma samples, taken from all three goats in Group 5 and from all three goats in Group 8 for DB 1192, when treated with four applications of 2.5 mg/kg or four applications of 1.25 mg/kg, respectively, against time.



Moreover, pharmacokinetic profiles were created to show the correlation between the different lead compounds, compared with the standard compound diminazene, at the treatment doses of 2.5 mg/kg (depicted in *Figure 7.6*) and at 1.25 mg/kg (depicted in *Figure 7.7*). Both *Figures 7.6* and *7.7* clearly show the high concentration of diminazene in the plasma of the goats after the final compound injection was given, followed by its rapid decline as time increases. In comparison, the relatively low compound concentrations of DB 1192 within the goats' plasma can also be clearly seen on the pharmacokinetic profile. Although DB 75 and DB 867 did not produce such high compound concentrations in contrast to diminazene at 5 mg/kg, the curves of best fit for these two compounds remain above that of diminazene.

Table 7.2. Pharmacokinetic parameters determined for the standard compound (diminazene) at 5 mg/kg and each of the three lead diamidine compounds (DB 75, DB 867 and DB 1192) at 2.5 mg/kg, used as a treatment schedule for *T. evansi* experimentally infected goats.

Parameter	Diminazene *(4 x 5 mg/kg)*	DB 75 *(4 x 2.5 mg/kg)*	DB 867 *(4 x 2.5 mg/kg)*	DB 1192 *(4 x 2.5 mg/kg)*
C_{max} (ng/ml)	≥ 8637	≥ 2530	≥ 3310	≥ 1563
T_{max} (hrs)	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
AUC	193589	358215	360895	9861
AUMC	13683170	33469105	32471327	378645
MRT (hrs)	70.7	93.4	90.0	38.4
K_{elim} (hrs^{-1})	0.01	0.01	0.01	0.03
$T_{½}$ (hrs)	59.1	473.7	221.4	30.5

Table 7.3. Pharmacokinetic parameters determined for the standard compound (diminazene) at 5 mg/kg and each of the three lead diamidine compounds (DB 75, DB 867 and DB 1192) at 1.25 mg/kg, used as a treatment schedule for *T. evansi* experimentally infected goats.

Parameter	Diminazene *(4 x 5 mg/kg)*	DB 75 *(4 x 1.25 mg/kg)*	DB 867 *(4 x 1.25 mg/kg)*	DB 1192 *(4 x 1.25 mg/kg)*
C_{max} (ng/ml)	≥ 8637	≥ 3115	≥ 3663	≥ 836
T_{max} (hrs)	≤ 0.5	≤ 1.0	≤ 0.5	≤ 0.5
AUC	193589	394305	381604	4809
AUMC	13683170	35295790	33558186	251434
MRT (hrs)	70.7	89.5	87.9	52.3
K_{elim} (hrs^{-1})	0.01	0.01	0.01	0.02
$T_{½}$ (hrs)	59.1	279.3	202.5	37.0

Figure 7.6. Pharmacokinetic profiles showing the correlation between diminazene at 5 mg/kg and DB 75, DB 867 and DB 1192 at 2.5 mg/kg treatment doses, with the corresponding curves of best fit.

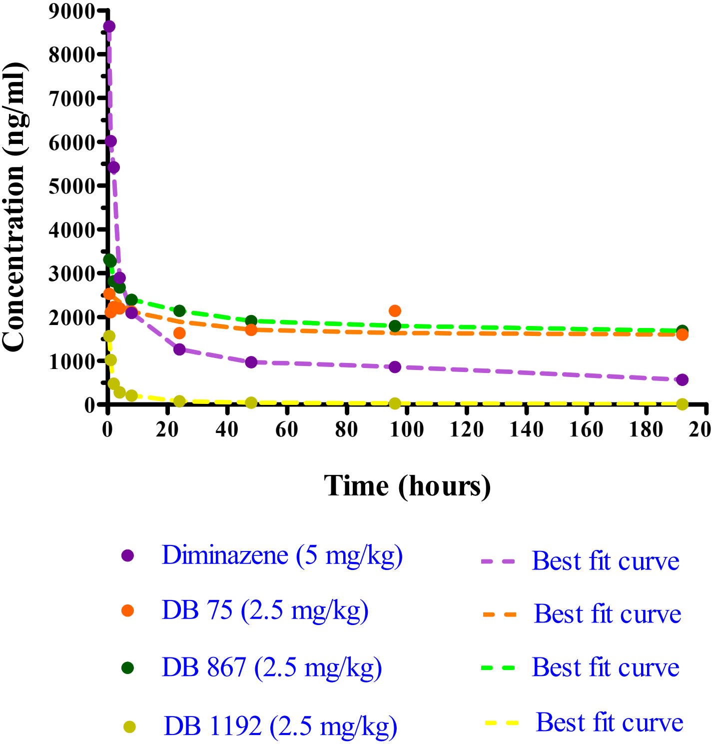
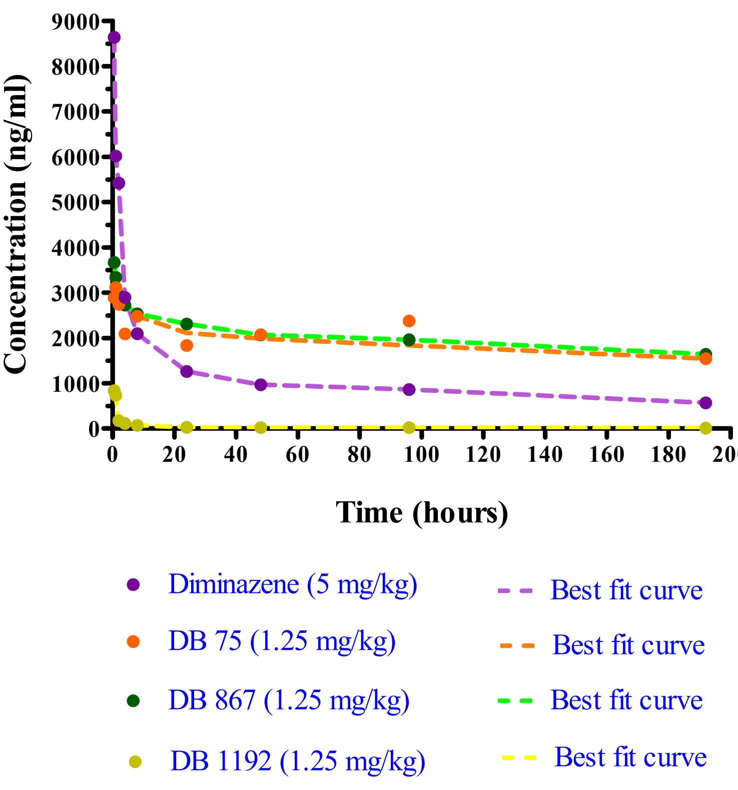


Figure 7.7. Pharmacokinetic profiles showing the correlation between diminazene at 5 mg/kg and DB 75, DB 867 and DB 1192 at 1.25 mg/kg treatment doses, with the corresponding curves of best fit.



Discussion

The aim of this pharmacokinetic study was to collect information for three lead diamidine compounds (DB 75, DB 867 and DB 1192) within an experimentally infected *Trypanosoma evansi* goat model. Thereafter, pharmacokinetic parameters were determined and the pharmacokinetic profiles for each compound, including the positive control (standard) compound, diminazene, were established by plotting the compound concentration values (in ng/ml) from the plasma samples against time. Based on these results, it is possible to understand how each compound underwent the processes of absorption, distribution and elimination after they were administered.

Absorption is an important process when intramuscular injections are given, which delays and reduces the magnitude of the peak compared to that seen with an intravenous dose. Therefore, all peak plasma concentrations (C_{max}) seen in this study are expected to be lower (and of course the time periods for T_{max}, longer) than those seen if the study was repeated using an intravascular route of compound application. In the case of intramuscular compound application, the time at which peak compound concentration occurs (T_{max} and C_{max}, respectively), some of the compound will remain at the absorption site and some will have already been eliminated; unlike an intravenous injection, where the entire dose enters the body immediately. However after such a peak, the C_{max} (intramuscular) will exceed the C_{max} (intravenous), due to the continual entry of the compound into the body from the muscles (*Ali et al., 2003b*). The compound concentrations obtained from the plasma samples provided information about the levels present, yet the first sample taken was at 0.5 hours after the last compound application. This limits the accuracy with which C_{max} and T_{max} can be defined, since had more samples been taken between T_0 (the time point at which the last compound application was given) and T_{30} (thirty minutes after T_0), then T_{max} may have been shorter (than 30 minutes) and C_{max} may have been higher than the values seen for each compound. Therefore, the values obtained for C_{max} and T_{max} in this study are attributed to either greater than or equal to that value ($\geq$), in the case of C_{max} and less than or equal to that value ($\leq$), in the case of T_{max}.

Once the compound has been absorbed, it needs to be distributed around the body to various organs and tissues. The distribution phase primarily accounts for the early rapid decline in plasma concentration. This decline relates to movement of the compound within the body and the rate a compound is distributed depends on factors such as plasma protein and DNA binding, tissue size and membrane permeability. It is not yet known exactly which

plasma proteins diamidines bind to, but basic compounds tend to bind with lipoproteins or alpha-1-acid glycoproteins. The absorption of compounds in solution from muscles is invariably perfusion rate-limited, so an increase in blood flow would speed up absorption (*Ali et al., 2003a*).

For compounds given extravascularly, the absorption process is critical in terms of therapeutic activity (*Elsheikh et al., 1997*). Increasing the dose produces a proportional increase in the plasma concentration at all times, unless the absorption half life or the availability (completeness of absorption) is changed. Therefore, if a dose is doubled, then at any given time, the amount of compound absorbed is doubled and with twice as much compound entering the body, twice as much compound needs to be eliminated. The difference between the amount of compound absorbed and the amount of compound eliminated is therefore also double. The reverse holds true for DB 1192, which was tested in the goats at 2.5 mg/kg and then at half this dose (1.25 mg/kg). The time for the peak should remain the same, but its magnitude should decrease proportionally with the dose. For DB 1192, T_{max} remained the same (≤ 0.5 hours), yet the C_{max} (≥ 836 ng/ml) was approximately half that of the higher dose (≥ 1563 ng/ml). In addition, the total area under the curve (AUC) should also be half, which is shown for the 1.25 mg/kg dose as 4'809 and for the 2.5 mg/kg dose as 9'861. However, DB 1192 failed to treat the infected goats at both doses tested. This can be explained when the compound concentrations in goat plasma are compared with the *in vitro* IC_{50} values for DB 1192. At 192 hours after the final compound injection was applied to goats treated with 2.5 mg/kg, the compound concentration in plasma was only 10 ng/ml. Through previous *in vitro* testing of DB 1192 against *T. evansi*, an IC_{50} value of 10.5 ng/ml was obtained after 72 hours. The minimal inhibitory concentration (MIC) of such a compound is at least four times greater than the IC_{50}, so for DB 1192, the estimated MIC (at 72 hours) would be around 42 ng/ml. To establish curative efficacy of an infected animal, the level of the compound in plasma must exceed the MIC for a certain time period. If the compound level in plasma falls below the MIC threshold too quickly, the compound provides only sub-therapeutic concentrations, which in turn will be insufficient to remove the parasites. At 72 hours, the compound concentration in the plasma (of the infected goats) was only 32 ng/ml. This value is already below the MIC threshold to ensure successful therapeutic activity of DB 1192. In fact, at 24 and 48 hours, the compound concentrations in goat plasma were 78 and 40 ng/ml, respectively. This shows that between 24 and 48 hours, the level was already below the MIC level required. In addition, 42 ng/ml is the MIC for 72 hours, which increases as time decreases; therefore the MIC at 48 hours alone would be much higher than 42 ng/ml and

probably is larger still than the mere 78 ng/ml seen at 24 hours. Thus, there is strong and clear evidence why DB 1192 could not ensure curative therapeutic activity at 2.5 mg/kg against goats experimentally infected with *T. evansi*. Furthermore, this relates to the lower dose of DB 1192 tested (1.25 mg/kg), where compound concentrations in goat plasma at 72 hours were only 18 ng/ml. Additional information demonstrates the compound concentration levels in plasma for 8, 24 and 48 hours to be 69, 23 and 18 ng/ml, respectively. Again with a 42 ng/ml value for the MIC of DB 1192 at 72 hours, the low compound concentration value of 69 ng/ml for 8 hours is far below the required therapeutic threshold.

For the standard control compound (diminazene), the *in vitro* IC_{50} value obtained for 72 hours is 12.5 ng/ml, with a corresponding MIC of 50 ng/ml. At 72 hours after compound treatment of 5 mg/kg doses to infected goats, the concentration of diminazene in goat plasma was 1112 ng/ml, which is a 22-fold greater concentration than the 50 ng/ml MIC value. Even after 192 hours, the diminazene concentration in goat plasma was 568 ng/ml, which still remains well within the therapeutic range; hence all goats treated with the standard compound were effectively cured. A similar pattern was seen in DB 75 at 2.5 mg/kg and 1.25 mg/kg, where the plasma concentrations derived at 72 hours were 1923 ng/ml and 2223 ng/ml, respectively. DB 75 has an IC_{50} of 2.3 ng/ml at 72 hours and an MIC of 9.2 ng/ml. At 192 hours, the plasma concentrations of DB 75 for both the high and low doses were 1600 ng/ml and 1540 ng/ml, respectively and well within the therapeutic range. Treatment of infected goats with DB 75 at both doses provided an effective cure.

Lower C_{max} values were seen for the higher 2.5 mg/kg dose for the compounds, DB 75 and DB 867. Both these compounds appear to accumulate within the body, as determined through their long half lives. If accumulation is high, then these compounds probably have a large affinity for tissue-binding. Since no differences were seen between the high (2.5 mg/kg) and low (1.25 mg/kg) doses tested, it may be that a point of saturation within the body has occurred at a specific compound dose. If a higher dose is administered, a larger amount of compound will be absorbed and bound to tissue, yet the amount which is unbound and free in the body may be the same amount, irrespective of whether the high or low dose is given. However, it is difficult to support such a theory with only plasma concentration data and would therefore require more detailed analysis and in-depth research into the accumulation and tissue-binding properties of diamidines in general.

In the case of DB 867, the *in vitro* IC_{50} at 72 hours was calculated as 1.7 ng/ml and the MIC as 6.8 ng/ml. Both the 2.5 mg/kg and 1.25 mg/kg DB 867 treated goats demonstrated compound concentrations in plasma at 72 hours of 1852 ng/ml and 2014 ng/ml, respectively.

After 192 hours, the compound concentration levels were still within the therapeutic range, being 1683 ng/ml and 1637 ng/ml, respectively. Goats treated with the higher dose (2.5 mg/kg) of DB 867 produced a curative efficacy, whereas goats treated with the lower dose of 1.25 mg/kg were seen to relapse after 4.5 months post treatment.

The phenomenon of relapse cases in both human and animal trypanosomiasis is not fully understood and many factors, such as drug resistant strains, reinfection or insufficient drug levels at a given site of the body, can be attributed as possible explanations for this. One aspect to consider would be the blood supply to various tissues, organs and compartments within the body. If trypanosomes have an ability to enter compartments or specific tissues (for example, cartilage), which have a reduced blood flow passing these areas, then insufficient drug levels may permeate these compartments/tissues, hence "hidden" trypanosomes will not receive the full therapeutic (trypanocidal) levels of the drug, enabling them to survive. Thereafter, once the drug concentrations within the body have been eliminated completely, these trypanosomes would then be free to reinvade different tissues and body compartments, thus building up a new population, which would produce the high parasitaemia observed in the goats after 4.5 months post treatment with DB 867 at 1.25 mg/kg. Whether other physiological factors play a vital role in this phenomenal situation would have to be further investigated.

Acknowledgments

The authors wish to acknowledge the hard work and effort invested by the staff of the Hospital Clinicó Veterinario in Arucas, Gran Canaria and for the kind use of their veterinary facilities. Furthermore, sincere gratitude is extended to James E Hall for help with the pharmacokinetic analysis.

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Fluorescent uptake of DB 75, DB 867 and DB 1192 in *Trypanosoma evansi*

Kirsten Gillingwater

Parasite Chemotherapy, Swiss Tropical Institute, Socinstrasse 57, 4002 Basel, Switzerland

Working paper

Abstract

How a drug can be taken up into parasitic cells, without harming host cells and without causing adverse effects, is important when designing new drugs to combat infection. Diamidines can be transported into trypanosomes using the same carrier proteins as those necessary for trypanosomal salvaging of purines. Once inside a parasitic cell, the bound complexes and target areas affected by the drug can reveal its mode of action. Even though the exact mode of action for novel diamidine compounds is not currently understood, they are believed to bind to the minor groove of AT-rich DNA sites and inhibit transcription.

Based on the efficacy of three lead diamidine compounds (DB 75, DB 867 and DB 1192) against *Trypanosoma evansi*, an investigation into their natural fluorescent properties was carried out. The rate of uptake and the areas within the cell, where the compounds bound and accumulated, could be visualised using fluorescent microscopy. The uptake of the compound DB 1192 took longer, than the uptake of the compounds DB 75 and DB 867. Nevertheless, all compounds demonstrated a similar pattern of uptake, whereby blue fluorescence occurred first in the kinetoplast, followed by the nucleus and then the acidocalcisomes. Thirty minutes after incubation, the acidocalcisomes gave off a yellow fluorescence for the compounds DB 75 and DB 867. The slower uptake of DB 1192 may shed some understanding as to why this compound produced the least therapeutic activity against *T. evansi* infection in mice and goats.

Keywords: Fluorescence; diamidines; *Trypanosoma evansi*; drug uptake.

Introduction

When discovering or designing new drugs, the mechanisms involved in getting that drug into a parasite is an important factor for eventually destroying the parasitic organism. In addition, the ideal outcome of a new drug would enable destruction of the parasite with minimal or no harm towards host cells. Nowadays, medicinal chemists have the ability to alter synthesised compounds, providing compounds with favourable properties, such as compounds with greater lipophilic features, enabling them to cross cell membranes easier, or

to provide compounds with less toxicity, hence limiting the adverse effects seen when host cells are harmed.

The diamidine drug, pentamidine, has found great use in the treatment of *Pneumocystis carinii* pneumonia, leishmaniasis and early stage human African trypanosomiasis, but is hindered by toxic effects such as hypotension, hypoglycaemia and renal failure (*Pepin and Milord, 1994*), to name a few. The uptake of pentamidine into trypanosomes relies on three specific transporters (*de Koning, 2001*), the high affinity pentamidine transporter (HAPT), the low affinity pentamidine transporter (LAPT) and the P2 transporter (coded by a *TbAT1* gene) (*Mäser et al., 1999*), also responsible for the uptake of adenosine and adenine. Since trypanosomes cannot produce purines *de novo*, they must salvage them from the host and actively transport them into the cell. Drugs, showing a similar motif (especially diamidines and melaminophenyl arsenicals) are also actively taken up by the P2 transporter (*Carter et al., 1995*; *de Koning and Jarvis, 1999*; *de Koning et al., 2004*). Recent investigations however, suggest there may be additional transporters involved, such as a P1 transporter (*de Koning, personal communication*), but it is not yet known to what extent these transporters may be involved in drug uptake.

Another interesting factor about drugs (whether new or old) is their mode of action. This involves the respective target, which the active part of a drug will attack and thus cause the ultimate destruction of the parasite, either directly or indirectly. Diamidines are known to favour AT-rich regions and can bind to the minor groove of DNA, before exerting their effect on the transcription process, either directly or indirectly by inhibiting DNA dependent enzymes, for example, nucleases or topoisomerases (*Wilson et al., 2005*). All the same, the exact mode of action of these compounds has not been fully elucidated.

Since the three selected lead diamidine compounds, DB 75, DB 867 and DB 1192 were chosen based on their *in vitro* and *in vivo* efficacy and low or no preliminary toxicity in mice and goats against *Trypanosoma evansi*, the causative agent of a worldwide animal pathogenic wasting disease called surra, an attempt was made to determine whether all three compounds contain similar fluorescent properties and whether they are taken up into *T. evansi* in the same way. Once taken up by the trypanosomes, a clearer insight into where these compounds bind or accumulate may be additionally revealed.

Materials and Methods

Trypanosomes

Trypanosoma evansi (STIB 806K) is a kinetoplastic strain and was isolated from a water buffalo in China in 1983. This strain was used to perform the fluorescent uptake studies.

Lead diamidine compounds

The selected diamidine compounds were originally designed and synthesised by the chemist, David Boykin. The compounds DB 75, DB 867 and DB 1192 were used as the test compounds in this fluorescent uptake study, based on their previous *in vitro* and *in vivo* activity within a mouse model and *in vivo* activity within an experimentally infected *T. evansi* goat model.

Stock solutions

Stock solutions of 10 mg/ml of each compound were prepared, dissolved in DMSO and then stored frozen at -20 °C. From these stock solutions, further compound dilutions were made for use in the fluorescent uptake study, together with culture medium as a solvent. Compound dilutions were made fresh on the day of each experimental procedure.

Culture medium

Bloodstream form trypanosomes were cultivated in Minimum Essential Medium (MEM) (powder, GIBCO/BRL, No: 11400-033) with Earle's salts, supplemented with 25 mM HEPES, 1 g/l additional glucose, 2.2 g/l $NaHCO_3$ and 10 ml/l MEM non-essential amino acids (50x concentration). The medium was then further supplemented by adding 1 % of a 2-mercaptoethanol stock (14 µl of 12 mM 2-mercaptoethanol was diluted in 10 ml of distilled water), 1 % of a stock consisting of 100 mM sodium pyruvate and 50 mM hypoxanthine and 15 % heat inactivated horse serum, according to *Baltz et al., 1985*. The complete medium is called Baltz MEM (BMEM).

Fluorescent uptake studies

Microscopic slides were labelled and prepared ready for the study. The slides were cleaned beforehand with 70 % ethanol to remove any dust residue. Trypanosome cultures previously cultivated, with an approximate parasite concentration of 10^5/ml, were taken and 99 µl of these cultures were mixed with 1 µl of a 1 mg/ml solution of the lead diamidine compounds, DB 75, DB 867 or DB 1192, respectively. The stopwatch was immediately started as soon as the compound was mixed with the trypanosome culture.

Thereafter, 5 µl droplets of each incubated culture were placed onto clean microscopic slides and a cover slip placed on top. This cover slip was then sealed onto the microscopic slide using Vaseline. This was done for the following time points after initial incubation with DB 75, DB 867 or DB 1192: 1, 5, 10, 20 and 30 minutes. The slides were then observed using a Leica DC200 fluorescent microscope (Leica Microsystems AG, Glattbrugg, Switzerland) at a magnification lens of x 50 with oil immersion and pictures were taken using an imaging programme (IM500, Version 1.20, Image Manager from Leica IM) for each time point and for each diamidine compound.

Results

The selected lead diamidine compounds DB 75, DB 867 and DB 1192 all demonstrated fluorescent properties, allowing them to be visualised under a fluorescent microscope at an excitation wavelength of 350 nm. Photo images were taken for each diamidine compound at 1, 5, 10, 20 and 30 minutes after incubation with *Trypanosoma evansi*, STIB 806K. These images can be seen in *Figure 8.1* for the compound DB 75, *Figure 8.2* for the compound DB 867 and *Figure 8.3* for the compound DB 1192.

All three compounds showed a similar pattern as they were taken up by the trypanosomes. The kinetoplast structure showed blue fluorescence first, followed by blue fluorescence within the nucleus. These two structures (kinetoplast and nucleus) then suddenly fluoresced at a peak point, after which small, circular structures within the cytoplasm gave off a blue fluorescence. These small, circular structures are known as acidocalcisomes. Thereafter a general equilibrium of fluorescence was seen throughout the whole trypanosome, before the acidocalcisomes gave off a yellow fluorescence, whilst the nucleus and kinetoplast remained

faintly blue. Although the process was similar for all three lead diamidine compounds, the times at which the various stages occurred were different between DB 1192 and the remaining two compounds.

Figure 8.1. Fluorescent images taken for *T. evansi* incubated with DB 75 at the time points of 1, 5, 10, 20 and 30 minutes.

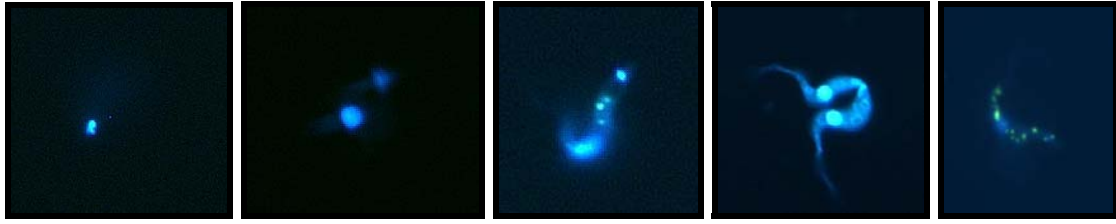

Figure 8.2. Fluorescent images taken for *T. evansi* incubated with DB 867 at the time points of 1, 5, 10, 20 and 30 minutes.

Figure 8.3. Fluorescent images taken for *T. evansi* incubated with DB 1192 at the time points of 1, 5, 10, 20 and 30 minutes.

The kinetoplast was seen fluorescing at one minute after incubation in *Figures 8.1* and *8.2* for the compounds DB 75 and DB 867, respectively. The image for DB 1192 in *Figure 8.3* at one minute shows no kinetoplast fluorescence and it is only after five minutes that an appearance of the kinetoplast is finally seen. By this time, DB 75 and DB 867 already demonstrated strong fluorescence within the kinetoplast and nucleus. Again this stage happened slightly slower in DB 1192 and occurred after ten minutes incubation only. The acidocalcisomes appear after ten minutes incubation with DB 75 and DB 867, yet with DB

1192, they were not so prominent. After twenty minutes incubation, there was an immense fluorescence in the nucleus of the trypanosomes, seen more clearly in DB 1192 on the thirty minute image rather than the twenty minute photograph. Ultimately the acidocalcisomes produced a yellow fluorescence with DB 75 and DB 867 after thirty minutes, which was not yet evident in DB 1192 at the same time point.

Discussion

The aim of this fluorescent uptake study was primarily to determine whether DB 75, DB 867 and DB 1192 displayed sufficient fluorescent properties to visualise and track the compounds and whether the uptake of these three selected lead diamidines could shed some light on their mode of action.

Indeed, all three compounds did fluoresce at an excitation wavelength of 350 nm. In addition, the uptake of these compounds could be observed in *Trypanosoma evansi* at various time points, after incubation with DB 75, DB 867 or DB 1192, respectively. Both, DB 75 and DB 867 appeared to be fairly similar during the uptake process, whereas DB 1192 was much slower, showing longer time delays between itself and the other two compounds. The P2 transporter, which has been reported to be involved in the active uptake of many diamidines and melaminophenyl arsenicals, remains one of the most predominant purine transporters and is responsible for the majority of DB 75 uptake visualised in trypanosomes. Since DB 867 is fairly similar to DB 75, both in structure and in efficacy against *T. evansi* (*in vitro* and *in vivo*), it would be reasonable to expect DB 867 to act in a comparable way to DB 75. From the fluorescent images produced, there appear to be no significant differences between DB 867 and DB 75 uptake. Whether the slower DB 1192 uptake into the parasite is by another predominant transporter, rather than by the *TbAT1*-coded P2 nucleoside transporter, remains to be further investigated.

Another possibility for the time delays seen in DB 1192 could result from compound binding complexes. As DB 75 and DB 867 are taken up into the trypanosome, they rapidly bind to AT-rich regions within trypanosomal DNA. Such areas include organelles such as the kinetoplast and nucleus. Once bound to these structures, the drugs are stored and no longer freely available in the cell cytoplasm. This allows more drug compound to be actively pumped in, until an equilibrium is obtained. The large affinity of DB 75 to bind to the minor

groove of trypanosomal DNA is believed to be as a result of non-bonded interactions between the furan active group and the walls of the AT-rich minor groove. If this is seen with DB 75, a diphenyl furan, then a similar binding could be occurring with DB 867, an aza-furan, hence explaining the strong fluorescence seen in *Figure 8.2*.

In the case of DB 1192 (an indole, however) these non-bonded interactions might not be so potent, thus reducing the ability of DB 1192 to bind strongly to the minor groove of trypanosomal DNA, resulting in less compound taken up and stored in the organelles, hence a slower activity against the parasite, which in turn could account for the extra time needed before fluorescence began to appear. Furthermore, if DB 1192 takes longer to cross over the cell membranes of these organelles and form these non-bonded DNA complexes, then the compound will take much longer before it can exert its biological activity and hence its therapeutic potential. This may be a suggestion as to why DB 1192 showed decreased efficacy against *T. evansi* in previous *in vivo* studies compared to DB 75 and DB 867.

Fluorescence for these three selected lead diamidines was not just detected in DNA-containing organelles (blue fluorescence), yet also in small, circular structures called acidocalcisomes. Over time, these acidocalcisomes changed from blue to yellow fluorescence. Acidocalcisomes are acidic calcium storing organelles, characterised by their high density, acidic nature and large content of pyrophosphate, polyphosphate (polyP), calcium and magnesium (*Moreno and Docampo, 2003*). They have been found in many microorgansims, ranging from both prokaryotic to eukaryotic types, yet are not present in mammalian cells. Acidocalcisomes are believed to be involved in certain metabolic pathways and tend to be randomly distributed, usually located at the anterior end of trypanosomes (*De Souza, 2002*). Although acidocalcisomes have also been reported to be involved in the osmoregulation of *Trypanosoma cruzi* (*Rohloff et al., 2004*), their function in *T. evansi* has not yet been fully investigated.

Acknowledgements

The author wishes to thank Sacha Schneeberger for additional assistance and technicalities with the experimental procedures, for help with the photo imaging and for the introduction into the fluorescent microscope.

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General Discussion

The overall aim of this thesis was to investigate novel diamidine compounds against the animal pathogenic protozoan, *Trypanosoma evansi*, to determine whether any had the potential to become clinical candidates and hence offer a more effective, less toxic, cheaper alternative to the current standard drugs available.

Firstly, a panel of drug sensitivities was established from previously isolated *T. evansi* and *Trypanosoma equiperdum* strains, to understand the extent of drug resistance occurring for the four standard drugs currently in use. The results revealed that reduced drug sensitivities are present in many of the strains tested and that a new drug against infection is desperately needed. Since debates are still open as to whether *T. equiperdum* is a separate species or may be misidentified *T. evansi* strains (*Claes et al., 2003*; *Claes et al., 2006*), both were tested in this case and an *in vitro* profile of drug sensitivities was created for future reference purposes.

Through *in vitro* screening of around 200 diamidine compounds, using cell viability and cytotoxicity assays, a large quantity of compounds could be tested in a fairly short period of time. A vast amount of reproducible information was generated for each respective compound from this process. Most importantly though, the selective criteria being sought after could be adjusted or modified to adhere to those specific requirements desired. Of all the compounds tested, the majority demonstrated activity against *T. evansi* (with IC_{50} values below 30 ng/ml), but selection was narrowed for lead identification through the cytotoxicity assays and the investigations with the knock out TbAT1 strain. As previously mentioned in the respective chapter, compounds relying less on P2 transportation would be advantageous considering the information we know about resistant strains (*Mäser et al., 1999*; *De Koning, 2001*). However, more research needs to be conducted on a molecular level to fully identify and elucidate all possible transporters and uptake mechanisms, which diamidines may use to enter trypanosomes.

From here, preliminary *in vivo* toxicity studies provided another source of crude selection and many compounds were removed based on their resulting acute toxic effects within a mouse model. Thereafter, the *in vivo* chemotherapeutic efficacy was investigated, allowing us to observe how these compounds behaved within a living system, especially in the presence of certain biochemical factors, which cannot be mimicked under *in vitro* conditions. Similarly to lead identification *in vitro*, the majority of the 48 diamidine compounds tested, were seen to be active against *T. evansi* infection in mice. Due to the

relatively low curative doses demonstrated by the four standard drugs against mice infected with *T. evansi* (between 2 mg/kg for diminazene and 0.0625 mg/kg for cymelarsan), the selective criteria, which are based on finding a superior compound to that of the standard drugs, were so stringently specific, it meant that many of the *in vivo* active compounds were not positively selected for further investigation in this project. It should be noted here however, that these compounds are not complete failures, since the curative treatment of infected mice with doses as small as 2 mg/kg are still incredibly active, especially when compared to other trypanosomal infections (such as with *Trypanosoma brucei rhodesiense*), where curative efficacy of infected mice can require treatment doses of up to 20 mg/kg. So, for the purposes of this project, only those diamidines demonstrating very low curative doses were optimised and further examined for pre-clinical assessment in larger animals.

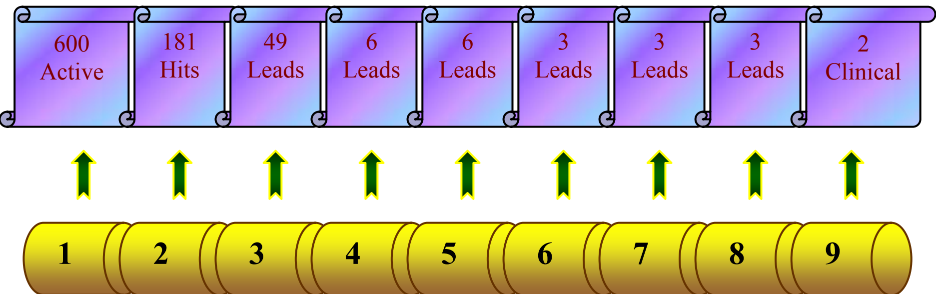
Six selected diamidine compounds were further assessed, based on *in vitro* and *in vivo* experiments. With treatment doses of just 0.5 mg/kg, given over four consecutive days, mice were effectively cured from *T. evansi* infection. This effect was also seen in different strains from various geographical locations. As *T. evansi* infection is found on the majority of the world's continents, a compound capable of exerting its curative efficacy on several strains can only benefit its marketable portfolio. So too can the costs involved in the manufacturing of a new drug. By reducing the costs involved in compound synthesis, be it in the raw materials or in the complex stepwise conditions utilised, the overall cost of the developed compound will also decline. This is critical for successfully targeting those poorer communities, economically dependent on their livestock, yet unable to find the means to purchase over-priced drugs.

This project enabled drug discovery against *T. evansi* infection to enter pre-clinical (and in some aspects clinical phase) trials. A higher animal model provides specialised data on the toxicological, biological and pharmacological qualities of the chemical compound(s) being analysed and developed. To follow the experimental processes carried out in this project, a PhD pipeline (*Figure 9.1*) was generated to reveal the different stages covered, with their related outcomes. The risky and unstable business of drug discovery and development is clearly shown in this PhD pipeline, where we began with 600 active compounds, yet only three progressed to pre-clinical evaluation (DB 75, DB 867 and DB 1192).

A goat model was chosen to act as the higher animal model against *T. evansi* infection, partly due to the size of such an animal and partly due to the biology behind this disease. To synthesise a specific amount of the test compound(s) takes time and financial resources, which were tailored for the resources available in this project. To undertake such pre-clinical

trials within cattle or camels for example, would require kilogramme quantities of test compound(s), requiring in turn approximately one and a half years for the manufacture of one compound alone. Coupled with the cost of performing, maintaining and analysing such a cattle/camel model, one can begin to understand why pharmaceutical entities are usually at the forefront of such experimental investigations. Nevertheless, within a three month period, a total of two grams were synthesised for each test compound, which was sufficient to perform all the goat-related investigations described within this project.

Figure 9.1. A PhD project pipeline depicting the different levels of drug discovery and development, according to the investigations carried out, with the outcomes for diamidine compounds, as potential clinical candidates against *T. evansi* infection.



1. Original compounds (**Early explorative discovery**)
2. *In vitro* screening (**Lead identification**)
3. *In vivo* (mouse) preliminary toxicity (**Lead identification**)
4. *In vivo* (mouse) curative efficacy (**Lead identification**)
5. *In vivo* (mouse) strain sensitivity (**Lead optimisation**)
6. Cost evaluation of synthetic manufacture (**Lead optimisation**)
7. *In vivo* (goat) toxicity trials (**Pre-clinical trials**)
8. *In vivo* (goat) pharmacokinetic study (**Pre-clinical trials**)
9. *In vivo* (goat) curative efficacy (**Clinical phase trials**)

Although many domestic and wild animals have been reported susceptible to *T. evansi* infection, some demonstrate acute infection, whilst others demonstrate a chronic progression of disease. Goats tend to develop the sub-clinical, chronic version of Surra, although severe clinical symptoms, acute disease and rapid death have also been accounted for in infected goats. Additionally, there have been no known attempts, where goats have been experimentally infected with *T. evansi*, followed by subsequent treatment with new chemotherapeutic compounds. Therefore, it was decided to use a goat model to determine the efficacy of selected diamidine compounds at treating not just experimentally infected goats,

but the chronic animal model of disease, as well as a potential reservoir host of *T. evansi* infection.

Before DB 75, DB 867 and DB 1192 could be investigated for curative efficacy, the toxicity of these compounds within goats needed to be analysed. Although the *in vivo* mouse model had shown no immediate or low toxic effects for these three compounds, drug metabolism in higher animals can be remarkably different and complex to that shown by mice. In addition, higher compound doses and multiple compound applications would be administered in the efficacy study, so the extent of accumulation by these compounds was not yet entirely known. One of the dangers presented by compound accumulation is the possibility of compound concentrations in plasma exceeding the therapeutic window threshold, thus entering the toxic range. No acute signs of toxicity were seen in goats tested with single or multiple applications of 4 mg/kg doses of DB 75, DB 867 and DB 1192. Various tissue and organ samples were collected for each compound from the respective goats and prepared for histological examination. These samples remain available for future examination, which could possibly reveal in-depth toxicological effects and findings, especially for the organs of compound elimination (liver and kidneys) and for critical regions, such as the site of injection.

Once the three selected lead diamidine compounds were considered safe to use in goats, an efficacy study was planned and conducted in the Canary Islands. *T. evansi* infection is currently endemic there with seroprevalances of 4.8 % to 9 % (1997-1999), after the initial importation of a male dromedary camel from West Africa in 1997. The primary task of finding a highly parasitaemic camel infected with *T. evansi*, enabled us to isolate the local strain and use it to experimentally infect the goats. In this way, no new strains of *T. evansi* were introduced onto the island and the infected goats closely represented the case of naturally infected animals from the area, due to the limited adaptation of the isolated strain through mice.

After the goats were infected, it was important that the control goats (receiving no compound treatment) revealed a constant parasitaemia, which is why the parasite concentration for infection (10^6) was quite high. This improved our probability of finding trypanosomes within the peripheral blood system, hence supporting the parasitological detection method used (HCT). However, three different diagnostic methods were applied in this efficacy study, to provide complete assessment of curative efficacy based on various arguments. Although demonstrating the causative agent through visual detection is an undisputable fact of infection, the absence of the parasite in this case, does not necessarily

imply cure or complete eradication. Therefore, a specific and sensitive method used to support the parasitological point of view usually involves the application of molecular DNA-based techniques, capable of detecting parasite DNA even if the parasite cannot be visually observed. The PCR technique, although new in this study, proved to be highly effective in demonstrating infection within the control goats (given no compound treatment), in goats where compound treatment failed completely (DB 1192 at 2.5 mg/kg and 1.25 mg/kg) and in goats which appeared to relapse at a later stage (DB 867 at 1.25 mg/kg). The positive detection of *T. evansi* shown by the PCR method was so sensitive, that in several situations, the PCR results became positive much earlier than the HCT did. However, this is expected since the PCR technique is more sensitive at detecting parasitic DNA in a small volume of blood, than the HCT can detect live parasites in the same amount of blood. The third diagnostic method used was the serological CATT / *T. evansi* test, to determine the antibody response of the infected goats to the RoTat 1.2 antigen. Like all serological tests, the CATT / *T. evansi* cannot differentiate easily between a recent or past infection. Although antibody responses in some diseases are considered an immunological benefit, they can be misleading in trypanosomal infections. Antibodies from previous trypanosome infections may not provide a protective effect, because trypanosomes have the ability to undergo antigenic variation. Consequently, the trypanosomes may display a new variant antigenic type (VAT) on their surfaces, which in turn will not be recognised by the previous antibodies, which may have been mounted against a different VAT. The CATT / *T. evansi* test was mainly used in the goat efficacy trial to assess the conditions of the goats during the follow up study.

Diminazene aceturate was used as a positive control in the goat efficacy study. Not only is it also a diamidine compound, but it is the standard compound used for treating goats infected with *T. evansi* at compound doses of around 5 mg/kg. To continue the main aim of the project and find a more efficient novel diamidine compound than the standard, the dosing of the test compounds (DB 75, DB 867 and DB 1192) were based on this figure. Hence, half and a quarter of the dose used by diminazene were selected, with the added knowledge that at 2.5 mg/kg and 1.25 mg/kg doses, these compounds should not exert any adverse or toxic effects within the goats, since doses of up to 4 mg/kg had been previously assessed in the goat toxicity trials. These figures also coincided well with the *in vivo* activity data from the mouse model, where approximately five-fold less compound doses of 0.5 mg/kg (of DB 75, DB 867 and DB 1192) had provided curative efficacy in *T. evansi* infected mice.

The results obtained from the goat efficacy study are very promising and two lead compounds have been selected as potential clinical candidates against *T. evansi* infection. The

most active compound, DB 75, was capable of curing the goats at 2.5 mg/kg and 1.25 mg/kg compound doses, just a quarter of the dose required by the standard compound, diminazene. In comparison, DB 867 was also very active and cured goats at 2.5 mg/kg compound doses (half that required by the standard compound). Treatment failures were seen with the third compound, DB 1192, at both compound doses tested and DB 867 relapsed at the lower compound dose of 1.25 mg/kg at 4.5 months post treatment.

The pharmacokinetic study carried out in parallel with the goat efficacy study, clearly demonstrated the presence of very low compound concentrations of DB 1192 in the plasma of the experimental goats. At these levels, DB 1192 was not within the therapeutic range and consequently could not effectively treat and cure the *T. evansi* infected goats. A different pattern was seen for DB 867, given at compound doses of 1.25 mg/kg however. The compound concentrations within plasma were high enough to remain within the therapeutic range, yet after nearly five months, these goats relapsed and infection was made apparent through the diagnostic methods used in the efficacy study. The phenomenon of relapse cases in trypanosomal infections can be caused by several factors, such as drug resistant strains, re-infection or insufficient drug levels at a given site of the body. The likelihood of re-infection in the goats treated with DB 867 at 1.25 mg/kg for example, was minimised in our study by keeping the infected control goats separate from the treated goats, plus all experimental goats were housed within fly-proof facilities. Furthermore, the potential of resistance for the *T. evansi* strain used to infect the goats is also considered unlikely, partly due to the fact that these three selected diamidine compounds have never before been investigated or released for use on a veterinary market. With no realistic possibility of prolonged exposure of this specific strain to these selected diamidines, the absence of a P2 transporter is also unlikely. Since diminazene is highly dependent on P2 transportation, one would also have expected to see reduced sensitivities for this compound, in both the mouse and goat *in vivo* studies previously performed. Since the pharmacokinetic profile of DB 867 demonstrated high compound concentrations in plasma, it seems doubtful that there were insufficient compound levels present. However, with a fairly long half life (~ 200 hours), DB 867 is believed to accumulate within tissues. Given that the amount of unbound compound is the active part, which correlates to the therapeutic activity, it may well be that DB 867 was present in insufficient concentrations in certain sites within the body, hence eventually leading to a possible relapse. It cannot be concluded for certain how or why such a relapse may have occurred in infected goats, treated with a low dose of DB 867, but with half the dose of that used for the standard

compound (diminazene), DB 867 remains one of the most active clinical candidates against *T. evansi* infection.

Future Perspectives

This PhD project has provided the basic scientific and investigative data for diamidines as potentially alternative chemotherapeutic agents against *T. evansi*. This class of compounds demonstrated higher efficacy against the parasite, than the majority of the standard drugs and were shown to be less toxic and economically beneficial for large scale production and manufacture. Two compounds, DB 75 and DB 867, have been brought through the discovery stages of early explorative discovery, lead identification and lead optimisation, into the developmental levels, involving pre-clinical trials and a pilot "proof of concept" efficacy study in a higher animal model. The scope of discovering and developing a new drug (from start to finish) cannot be covered completely within a PhD project. However, the promising results and analysis already performed and collected on DB 75 and DB 867, warrant their continued investigation, development and marketable potential as serious candidate molecules against *T. evansi* infection. Such a novel compound is desperately required on the international veterinary market.

Within the Canary Islands, a programme involving treatment of all infected camels and horses is currently underway, yet it does not take into account small ruminants, such as goats. Camel farms placed near to or within close proximity to goat herds presents a possible epidemiological catastrophe, especially if natural infection of *T. evansi* can occur in goats, which in turn can act as chronic reservoirs of the parasite. This was seen in our study, whereby one goat had previously been exposed to *T. evansi* infection and control goats could survive up to six months post-infection, with plenty of viable parasites circulating in the blood. Should such a programme successfully treat all infected camels and horses, the potential possibility of re-infection is incredibly high, if animals acting as disease reservoirs are not equally investigated and treated. Based on the results of our goat efficacy study, it would be advisable to use all three diagnostic methods when seeking out infected animals in such a programme, since parasitological methods can identify recent infections, serological methods can determine the immunological state of the animal and molecular based techniques can confirm the presence of parasite DNA in re-infected or relapsed animals at an early stage.

In addition, the situation of insect vectors as potential disease transmitters on the island should also be assessed and follow up studies should be conducted after the programme has terminated.

The pharmacokinetic goat study revealed that the pharmacokinetic profiles of DB 75 and DB 867 were quite similar, producing very high compound concentrations within plasma samples. With such long half lives for these two compounds, the extent of compound accumulation within the goats may be a critical factor required to fully understanding the absorption, metabolism and elimination of diamidines. Preliminary investigations, focused on compound uptake and accumulation, were performed for DB 1192, DB 867 and DB 75, using the compounds natural fluorescent properties. Detailed examination into the accumulative affinity of these compounds however to trypanosomes and to various tissues within the body, need to be performed to fully comprehend the processes going on between compound, parasite and host. The specifics for DNA and protein binding of diamidine molecules within the body, the distribution phases involved and the clearance of such compounds, are all areas of pharmacological interest, that could reveal much wanted knowledge about diamidines. In conclusion, the beneficial effects seen for DB 75 and DB 867 against *T. evansi* infection support the opinion that these two compounds have a stable advantage against other standard drugs and should be developed further for productive use on the international veterinary market.

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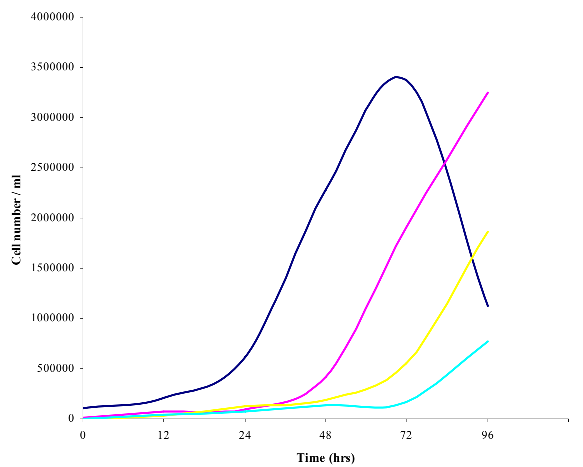
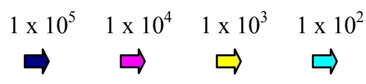
Appendices

1. Growth curves for several *Trypanosoma* sp. to determine optimal starting concentrations for different *in vitro* cell viability assays



Trypanosoma brucei rhodesiense

(STIB 900) strain

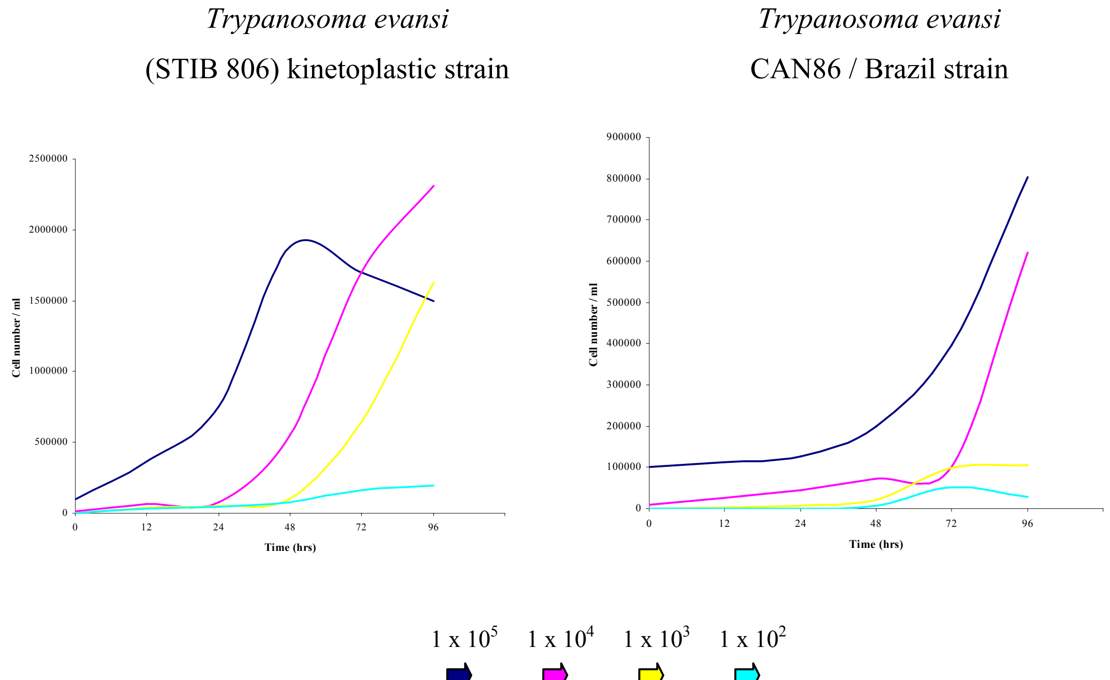


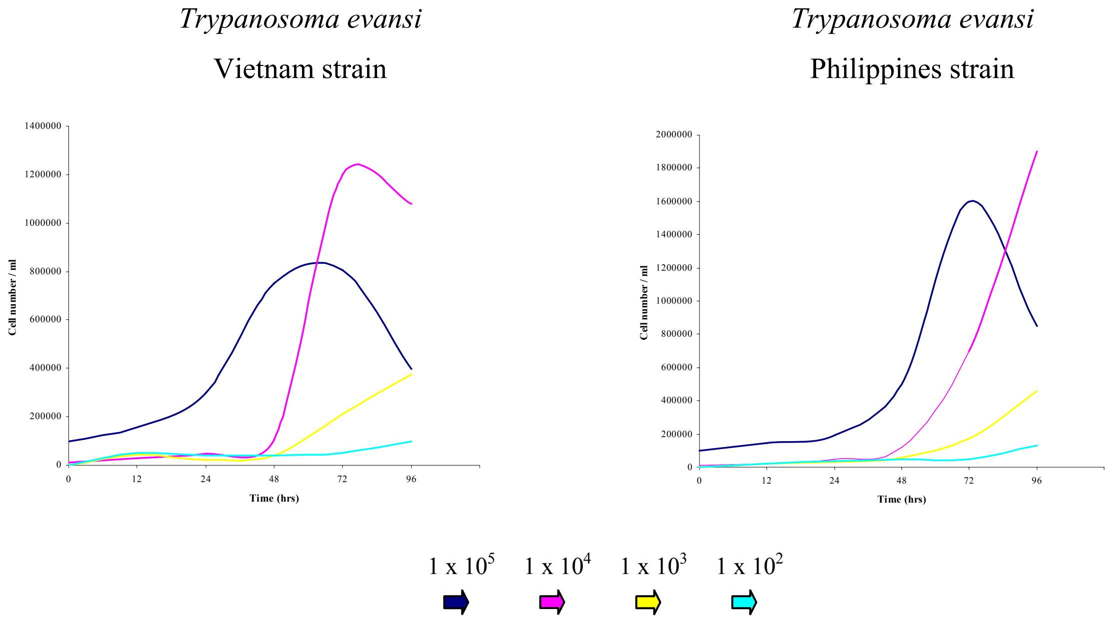
Trypanosoma evansi

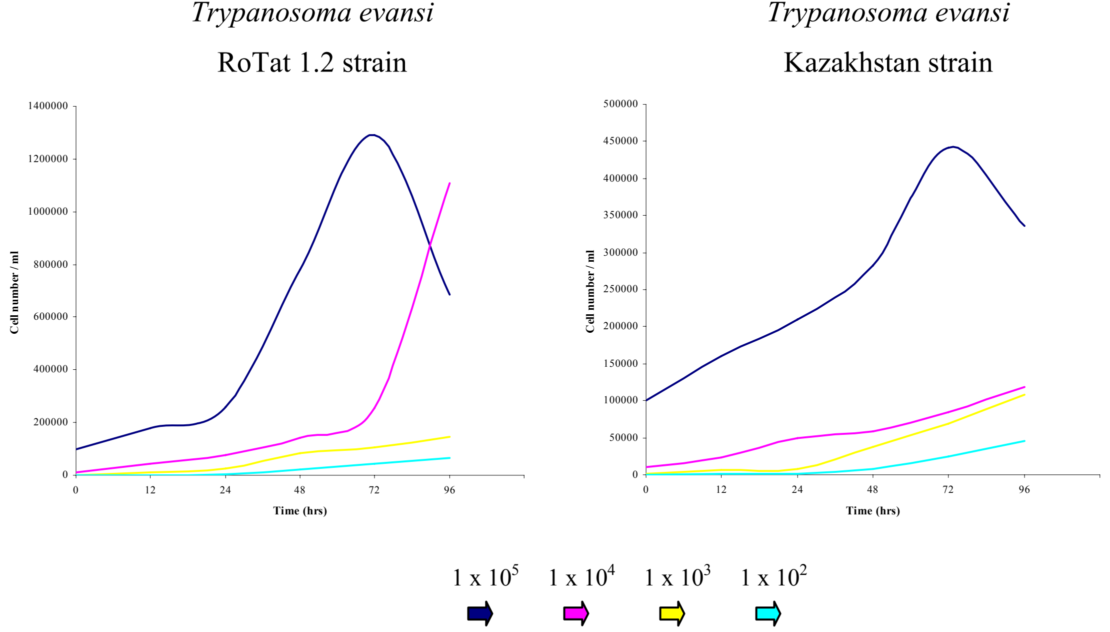
(STIB 806) kinetoplastic strain

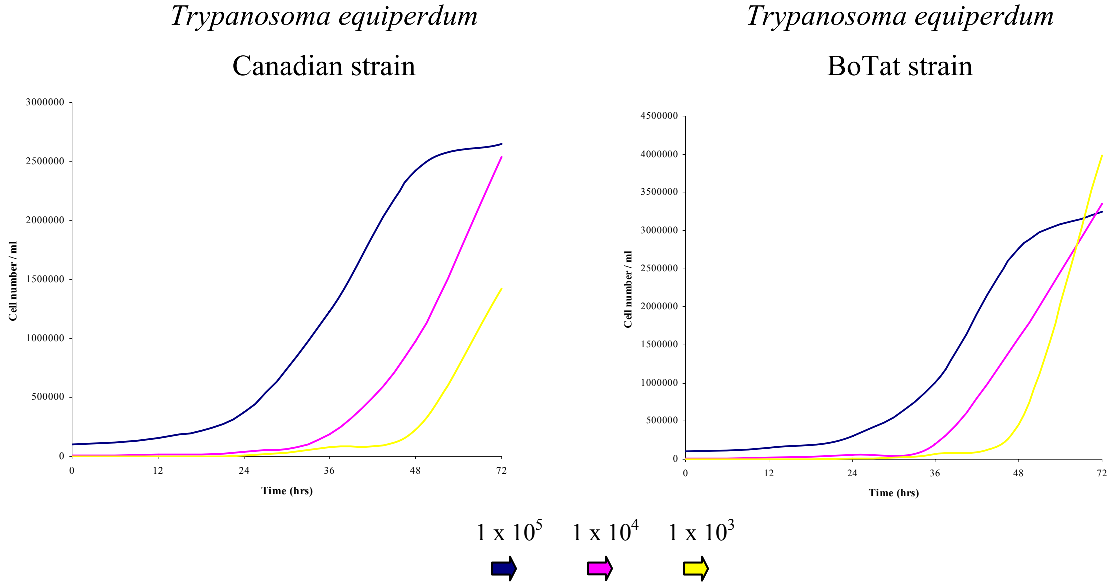
Trypanosoma evansi

CAN86 / Brazil strain

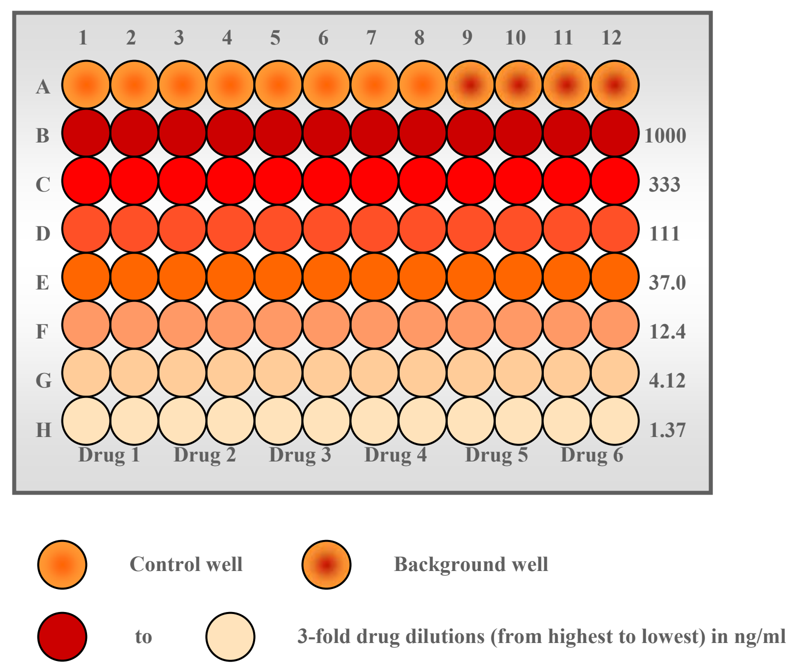



Trypanosoma evansi
Vietnam strain
Trypanosoma evansi
Philippines strain
Cell number / ml
Time (hrs)
Cell number / ml
Time (hrs)
1 x 10^5
1 x 10^4
1 x 10^3
1 x 10^2


Trypanosoma evansi
RoTat 1.2 strain
Trypanosoma evansi
Kazakhstan strain
Cell number / ml
Time (hrs)
Cell number / ml
Time (hrs)
1 x 10^5
1 x 10^4
1 x 10^3
1 x 10^2



2. Plate layout for the ^{3}H-hypoxanthine incorporation assay



3. SOP: *Trypanosoma brucei* species *in vitro* Assay (^{3}H-hypoxanthine incorporation)

Standard parasite strains: *Trypanosoma brucei rhodesiense* strains (STIB 900)
Trypanosoma evansi strains (STIB 806 K)
Trypanosoma equiperdum strains

Standard drugs: Suramin (10 mg/ml stock solution; start conc. 10 µg/ml)
Diminazene (10 mg/ml stock solution; start conc. 10 µg/ml)
Cymelarsan (10 mg/ml stock solution; start conc.100 ng/ml)
Quinapyramine (10 mg/ml stock solution; start conc. 100 ng/ml)

Standard conditions:

Medium: Minimum essential media (MEM)
Earle's balanced salt solution
Horse serum (inactivated)
Baltz solution (without Hypoxanthine)

Radioactive hypoxanthine: 500 µl ^{3}H-hypoxanthine stock + 500 µl EtOH + 24.5 ml medium (these 1 ml aliquots are stored at -20 °C, medium added fresh)

Plates: Costar™ 96-well microtitre plates

Incubation: 37 °C and 5 % humidified CO_2

Drug preparation: Compounds are prepared as advised by the supplier. If not specified, DMSO is used. Stock solutions of 10 mg/ml are made, which may be kept at room temperature or at -20 °C if an alternative solvent is used. (Since DMSO is toxic, care has to be taken not to exceed a final concentration of 0.5 % DMSO).

Assay Procedure:

Day 1

1. Into all wells of a 96 well microtitre plate, 50 µl of BMEM (containing no hypoxanthine) are added, with the exception of those wells in row B.

2. An additional 50 µl of BMEM (containing no hypoxanthine) is placed in the last four wells of row A (columns 9, 10, 11 and 12), to produce a total volume of 100 µl, as these wells act as a negative background.

3. Into the wells of row B, 75 µl of two times the highest drug concentration (previously calculated and mixed with BMEM medium containing no hypoxanthine) are placed according to the number of drugs being tested.

4. Per plate, six drugs can be tested - Drug 1 in columns 1 - 2, Drug 2 in columns 3 - 4, Drug 3 in 5 - 6, Drug 4 in 7 - 8, Drug 5 in 9 - 10 and Drug 6 in columns 11 - 12.

5. Then 25 µl of each well from row B are removed and using a multi-channel pipette, the drugs are diluted using a 3-fold serial dilution step, in that 25 µl from row B are mixed with row C, then 25 µl of row C are removed and placed into row D, mixed and again 25 µl removed and put into row E, and so on until row H is reached. The final 25 µl from row H are then discarded.

6. Using the CASY cell counter system, the correct trypanosome suspension can be achieved, accompanied by BMEM medium (containing no hypoxanthine). A total concentration of $2x10^6$ / ml of the required trypanosome suspension is calculated, where approximately 4.6 ml of medium are required for one 96 well microtitre plate.

7. Finally, 50 µl of this trypanosome suspension are then added to all wells, with the exception of the four negative control wells in row A, columns 9 - 12. Therefore, a total volume of 100 µl should be found in each well of the plate.

8. The plates are then placed into the incubator at 37 °C and 5 % humidified CO_2 for 24 hours.

Day 2

1. After the 24 hour incubation period, the plates are removed and 20 µl of a 1 µCi radioactive ^{3}H-hypoxanthine solution (freshly mixed with 24.5 ml of BMEM medium, containing no previous hypoxanthine) are added to all wells on the 96 well microtitre plate, including the four negative control wells.

2. The plates are then incubated for a further 16 hours under the same conditions.

Day 3

1. After a total incubation time of 40 hours, the plates are removed from the incubator and read in the cell harvester machine.

2. Steps for using the cell harvester can be found on the corresponding protocol sheet.

3. Once harvested, the plates are placed into the beta counter (using the protocol cassettes) and the machine is started, whereby the radioactive hypoxanthine counts are then evaluated and stored onto a floppy disk.

4. The data can then be analysed using a template sheet in a graphics programme (Microsoft Excel) and IC_{50} values determined and obtained for the drugs tested.

4. SOP: *Trypanosoma brucei* species *in vitro* Assay (Alamar Blue® assay)

Standard parasite strains: *Trypanosoma brucei rhodesiense* strains (STIB 900)
Trypanosoma brucei gambiense strains (STIB 930)
Trypanosoma evansi strains (STIB 806 K)
Trypanosoma equiperdum strains

Standard drugs: Melarsoprol™ (Main standard for STIB 900)
Suramin (10 mg/ml stock solution)
Diminazene (10 mg/ml stock solution)
Cymelarsan (10 mg/ml stock solution)
Quinapyramine (10 mg/ml stock solution)

Standard conditions:

Medium: Minimum essential media (MEM)
Earle's balanced salt solution
Horse serum (inactivated)
Foetal calf serum (inactivated) for *T. b. gambiense* strains
Human serum for *T. b. gambiense* strains
Baltz solution

Plates: Costar™ 96-well microtitre plates

Incubation: 37 °C and 5 % humidified CO_2

Drug preparation: Compounds are prepared as advised by the supplier. If not specified, DMSO is used. Stock solutions of 10 mg/ml are made, which may be kept at room temperature or at -20 °C if an alternative solvent is used. (Since DMSO is toxic, care has to be taken not to exceed a final concentration of 0.5 % DMSO).

Assay Procedure:

1. Into the wells of row H, add 75 µl of medium that contains two times the highest drug concentration desired. Per plate, 4 drugs can be tested (drug 1 column 1-3, drug 2 column 4-6, etc.)

2. Add 50 µl of medium at room temperature to rows A to G of a 96-well plate.

3. Serial drug dilutions are prepared by using a 12-channel multi-pipette. First, remove 25 µl from the wells of row H and place it into row G and mix well. Next, 25 µl are taken out of row G and placed into row F and so on until row B. The last 25 µl of row B are discarded. A serial dilution factor of 1:3 is thus obtained. The wells in row A serve as controls without drugs.

4. Thereafter, 50 µl of medium without trypanosomes are added to columns 3, 6, 9 and 12; these columns serve as background.

5. Dilute the trypanosomes to $4x10^4$ parasites / ml. The trypanosome density is adjusted with a Cell Analyser System (CASY, Schaerfe System) or by a count on the haemocytometer. (The trypanosome density used should be adjusted depending on the current growth characteristics of the corresponding cultures)

6. Per plate, allow for the use of 3.5 ml of the trypanosome stock.

7. Into the remaining wells, add 50 µl of trypanosome suspension.

8. The plates are then incubated for 69 hours at 37 °C and 5 % humidified CO_2.

Evaluation:

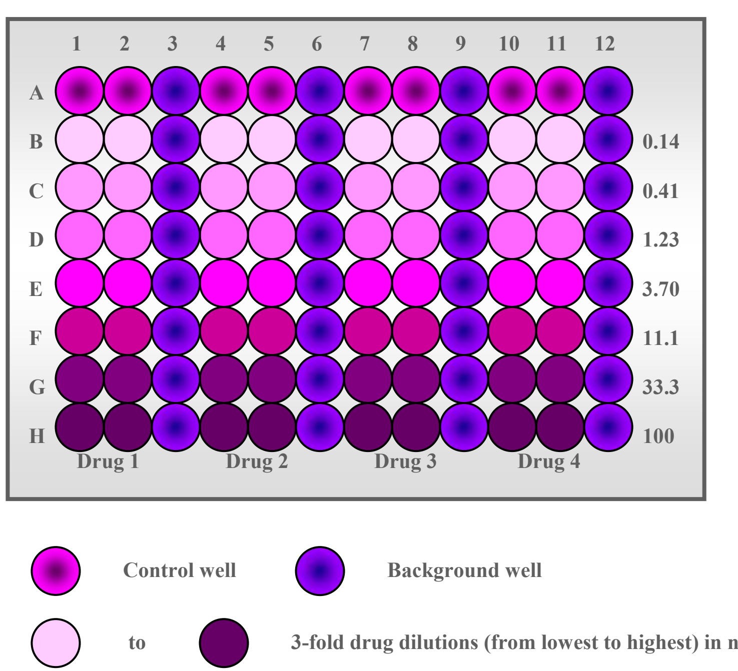
1. The plates are inspected under an inverted microscope to ensure that growth is normal. Additional information may be recorded, such as drug insolubility or contamination, etc.

2. Add 10 µl of the fluorescent dye Resazurin to each well and incubate for an additional 3 hours (until a subtle colour change is observed).

3. To determine an IC_{50} value, the plate is read at an excitation wavelength of 536 nm and emission wavelength of 588 nm.

4. Data are transferred into a graphics programme (SOFTmax Pro 3.1.2) and are evaluated to determine the IC_{50} values.

5. Plate layout for the Alamar Blue® assay



6. SOP: *In vitro* sensitivity assay for compound toxicity (Cytotoxicity assay)

Standard cell lines: L-6 (rat skeletal myoblast cells)
HT-29 (human bladder carcinoma)

Standard drugs: Suramin (10 mg/ml stock solution)
Diminazene (10 mg/ml stock solution)
Cymelarsan (10 mg/ml stock solution)
Quinapyramine (10 mg/ml stock solution)

Standard conditions:

Medium: Roswell park memorial institute media (RPMI 1640)
Foetal calf serum (inactivated)
L-Glutamine (1.7 µM)

Plates: Costar^{TM} 96-well microtitre plates

Incubation: 37 °C and 5 % humidified CO_2

Drug preparation: Compounds are prepared as advised by the supplier. If not specified, DMSO is used. Stock solutions of 10 mg/ml are made, which may be kept at room temperature or at -20 °C if an alternative solvent is used. (Since DMSO is toxic, care has to be taken not to exceed a final concentration of 0.5 % DMSO).

Assay procedure:

1. Add 100 µl of medium to wells of columns 3, 6, 9 and 12 of a microtitre plate. These wells serve as controls.
2. Next, 100 µl of a cell suspension of $4x10^4$ cells / ml is added into the remaining columns (1 and 2, 4 and 5, 7 and 8, and 10 and 11). Cells are allowed to attach overnight.
3. Per plate, allow for 6.5 ml of cell suspension to be used.
4. The following day, the medium is removed from row H (do this for half of the plates and go to step 4 and return to step 3 for the second half, so that the cells do not dry out).
5. Thereafter, 150 µl of medium containing the highest drug concentration is added to the wells of row H. Four drugs can be tested on one plate (drug 1 column 1-3, drug 2 column 4-6, etc.).

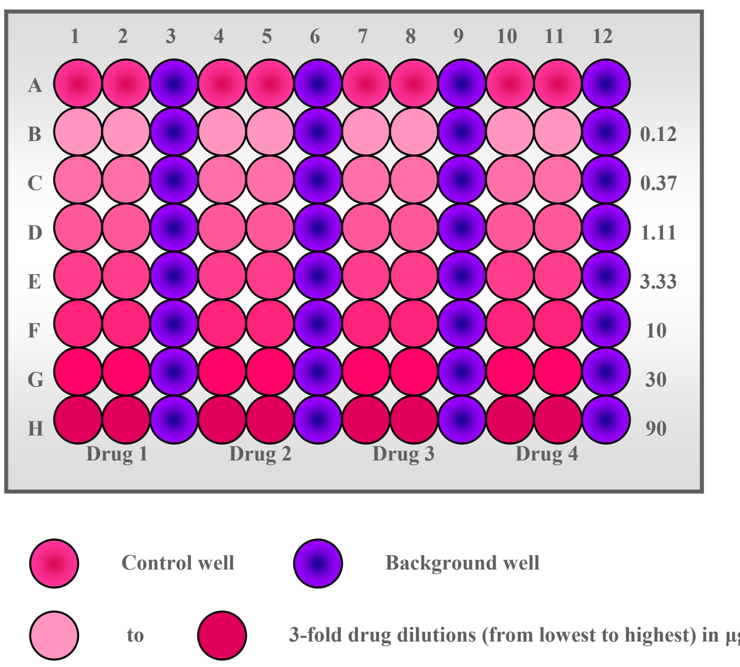
6. Serial drug dilutions are prepared by using a 12-channel multi-pipette. First, remove 50 µl from wells of row H and put into row G and mix well. Next, 50 µl are taken out of row G and put into row F and so on until row B. The last 50 µl of row B are discarded. A serial dilution factor of 1:3 is thus obtained. Wells in row A serve as control wells without drugs.

7. The plates are then incubated for 69 hours at 37 ºC and 5 % humidified CO_2.

Evaluation:

1. The plates are inspected under an inverted microscope to ensure that growth is normal. Additional information may be recorded, such as drug insolubility or contamination, etc.
2. Add 10 µl of the fluorescent dye Resazurin to each well and incubate the plates for another 2 hours (until a colour change is observed).
3. To determine an IC_{50} value, the plate is read at an excitation wavelength of 536 nm and emission wavelength of 588 nm.
4. Data are transferred into a graphics programme (SOFTmax Pro 3.1.2) and are evaluated to determine the IC_{50} values.

7. Plate layout for the L6 Cytotoxicity assay



8. SOP: *In vivo* preliminary acute toxicity test (mouse model)

Standard parasite strains: None

Standard conditions:

Mice: NMRI female mice, 22 - 25 g

Cages: Macrolon, type II cages

Maintenance: 22 °C and 60 - 70 % relative humidity
Pelleted food and water *ad libitum*

Test procedure:

Day 1: A mouse is injected (i.p.) with the compound being tested, at a starting concentration of 5 mg/kg. Thereafter, the doses then increase to 15 mg/kg, 30 mg/kg and 50 mg/kg with two hour intervals. The cumulative dose corresponds to the injection dose; hence 15 mg/kg has an accumulation dose of 20, 30 mg/kg of 50 and 50 mg/kg of 100, respectively. The mouse is constantly observed for any toxic or sub-toxic signs in order to determine the highest tolerated dose (HTD).

Day 2: The mouse is checked again after 24 hours for any signs of toxicity or sub-toxicity. If none are present, the highest tolerated dose can be recorded.

9. Raw data for the Alamar Blue® assay

Trypanosoma brucei rhodesiense (STIB 900):

Standard drugs

Drug	*Alamar Blue Assay for STIB 900*			
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
				Average
Suramin	85.28	91.946	50.215	**75.814**
Diminazene	3.067	2.357	2.917	**2.780**
Cymelarsan	1.544	2.838	1.443	**1.942**
Quinapyramine	0.209	0.566	0.193	**0.323**

Boykin compounds

Compound	*Alamar Blue Assay for STIB 900*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
75	0.858	0.824	**0.841**
103	10.354	7.938	**9.146**
201	8.465	9.29	**8.878**
210	10.725	8.437	**9.581**
211	1.98	5.833	**3.907**
217	2.968	3.523	**3.246**
242	7.815	9.385	**8.600**
262	4.606	4.584	**4.595**
320	1.129	3.051	**2.090**
325	5.668	2.633	**4.151**
346	3.23	1.61	**2.420**
351	0.895	0.502	**0.699**
417	1.89	3.923	**2.907**
427	2.047	6.156	**4.102**
484	1.561	1.523	**1.542**
497	13.43	11.043	**12.237**
508	1.96	2.795	**2.378**
544	3.263	3.553	**3.408**
545	4.355	3.257	**3.806**
560	1.765	1.814	**1.790**
609	6.03	6.411	**6.221**
690	1.837	1.783	**1.810**
746	9.129	8.964	**9.047**
763	6.238	6.923	**6.581**
773	4.279	4.777	**4.528**
820	1.414	1.281	**1.348**

Compound	*Alamar Blue Assay for STIB 900*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
828	5.634	5.769	**5.702**
832	3.332	3.821	**3.577**
841	7.388	10.046	**8.717**
846	10.983	10.457	**10.720**
849	4.01	3.498	**3.754**
850	5.305	5.353	**5.329**
851	13.474	12.389	**12.932**
853	13.427	13.918	**13.673**
866	2.022	2.798	**2.410**
867	0.639	0.943	**0.791**
877	0.137	0.319	**0.228**
902	6.439	8.425	**7.432**
911	1.371	3.336	**2.354**
930	1.487	2.069	**1.778**
935	5.001	9.653	**7.327**
943	3.069	5.898	**4.484**
945	2.199	3.498	**2.849**
988	3.563	3.37	**3.467**
989	3.187	5.764	**4.476**
994	1.049	1.146	**1.098**
1012	3.283	3.303	**3.293**
1016	4.304	9.536	**6.920**
1017	2.409	3.422	**2.916**
1019	0.245	0.583	**0.414**
1023	5.11	3.518	**4.314**
1044	3.901	9.542	**6.722**

Compound	*Alamar Blue Assay for STIB 900*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
1046	4.759	3.674	**4.217**
1049	11.493	6.901	**9.197**
1050	2.061	1.616	**1.839**
1052	4.808	9.566	**7.187**
1055	10.272	13.236	**11.754**
1065	0.716	0.91	**0.813**
1077	2.533	4.33	**3.432**
1114	4.767	3.471	**4.119**
1149	5.359	3.235	**4.297**
1152	0.464	0.461	**0.463**
1157	15.034	10.216	**12.625**
1159	10.162	10.153	**10.158**
1164	0.240	0.256	**0.248**
1165	1.357	1.212	**1.285**
1171	0.534	1.126	**0.830**
1172	5.743	3.489	**4.616**
1173	12.338	10.490	**11.414**
1177	2.104	3.378	**2.741**
1191	6.918	9.644	**8.281**
1192	0.371	0.504	**0.438**
1194	3.160	1.134	**2.147**
1197	8.828	11.051	**9.940**
1206	0.957	0.769	**0.863**

Compound	*Alamar Blue Assay for STIB 900*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
1210	1.353	1.862	**1.608**
1213	1.007	0.802	**0.905**
1214	7.693	4.159	**5.926**
1220	9.774	14.540	**12.157**
1228	3.539	2.229	**2.884**
1236	5.686	3.928	**4.807**
1237	10.145	5.648	**7.897**
1239	4.509	1.866	**3.188**
1242	6.510	4.925	**5.718**
1250	1.145	1.267	**1.206**
1253	3.435	7.936	**5.686**
1255	8.091	7.540	**7.816**
1258	13.051	6.046	**9.549**
1265	3.806	4.291	**4.049**
1266	9.534	5.834	**7.684**
1271	6.551	9.577	**8.064**
1272	5.667	4.578	**5.123**
1283	3.311	1.424	**2.368**
1288	0.809	0.969	**0.889**
1340	0.857	0.862	**0.866**
1342	6.045	5.933	**5.989**
1370	1.721	1.848	**1.850**
1371	16.107	16.485	**16.296**

Tidwell compounds

Compound	*Alamar Blue Assay for STIB 900*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
3 HXC 023	10.833	15.133	**12.983**
5 BGR 066	16.809	17.675	**17.242**
5 BGR 068	14.26	14.513	**14.387**
5 BGR 086	5.155	5.808	**5.482**
5 BGR 088	8.294	9.308	**8.801**
5 BGR 094	16.265	15.859	**16.062**
5 BGR 096	24.201	23.061	**23.631**
6 BGR 012	28.971	23.031	**26.001**
1 SMB 015	6.082	8.703	**7.393**
6 EVK 012	24.498	27.867	**26.183**
1 RRT 039	0.982	0.843	**0.913**

Compound	*Alamar Blue Assay for STIB 900*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
6 MAA 143	20.93	21.249	**21.090**
5 MAA 085	27.037	25.412	**26.225**
1 KAO 045	18.572	15.327	**16.950**
3 STL 057	6.687	2.064	**4.376**
5 MAA 083	22.187	20.661	**21.424**
5 MAA 101	12.502	7.095	**9.799**
5 MAA 121	16.287	15.078	**15.683**
5 MAA 123	25.068	23.381	**24.225**
5 MAA 137	20.632	23.671	**22.152**
6 MAA 025	3.639	2.622	**3.131**
2 SAB 087	21.735	22.95	**22.343**

Compound	*Alamar Blue Assay for STIB 900* IC$_{50}$ (ng/ml)	IC$_{50}$ (ng/ml)	IC$_{50}$ (ng/ml)
			Average
3 SMB 051	6.517	6.704	**6.611**
3 SMB 065	9.889	10.007	**9.948**
3 SMB 079	23.313	29.38	**26.347**
3 SMB 101	4.135	4.249	**4.192**
4 SMB 058	27.725	21.236	**24.481**
4 SMB 092	13.914	12.449	**13.182**
5 SMB 032	3.498	2.045	**2.772**
1 EVK 057	6.024	6.412	**6.218**
16 DAP 022	24.147	27.587	**25.867**
4 SAB 075	3.638	3.437	**3.538**
5 SMB 093	12.648	10.27	**11.459**
16 DAP 095	6.642	4.992	**5.817**
3 KEG 083	12.239	10.689	**11.464**
3 NAN 027	3.197	1.762	**2.480**
6 SAB 038	11.749	11.087	**11.418**
8 SMB 021	4.818	4.783	**4.801**
8 SMB 044	14.256	13.101	**13.679**
7 SAB 004	5.198	4.659	**4.929**
7 SAB 015	7.324	8.778	**8.051**
7 EVK 097	1.451	2.783	**2.117**
7 SAB 079	1.342	3.355	**2.349**
8 EVK 024	4.036	1.598	**2.817**
8 EVK 030	3.165	6.365	**4.765**
19 DAP 021	9.132	2.969	**6.051**
19 DAP 025	1.809	1.238	**1.524**
1 FMS 034	5.419	5.627	**5.523**
1 MCC 112	3.163	1.424	**2.294**
1 MCC 179	0.514	0.264	**0.389**
1 MCC 097	8.51	9.433	**8.972**
1 MCC 128	3.489	1.961	**2.725**
1 MCC 148	1.237	0.926	**1.082**

Compound	*Alamar Blue Assay for STIB 900* IC$_{50}$ (ng/ml)	IC$_{50}$ (ng/ml)	IC$_{50}$ (ng/ml)
			Average
1 MCC 184	6.185	3.435	**4.810**
10 SMB 038	13.595	6.599	**10.097**
0 DWB 082	3.852	3.062	**3.457**
0 MXB 736	28.29	28.168	**28.229**
0 MXB 767	25.261	15.984	**20.623**
0 MXB 864	10.634	7.801	**9.218**
10 SMB 084	29.875	33.909	**31.892**
11 SMB 003	8.752	12.282	**10.517**
19 DAP 075	13.774	11.659	**12.717**
19 DAP 085	18.873	14.45	**16.662**
19 DAP 089	9.638	11.796	**10.717**
20 DAP 001	10.484	6.223	**8.354**
19 DAP 079	15.955	8.725	**12.340**
8 SAB 066	1.677	0.996	**1.337**
8 SAB 068	1.08	0.65	**0.865**
8 SAB 072	5.54	3.637	**4.589**
20 DAP 055	6.502	5.714	**6.108**
20 DAP 059	5.198	3.105	**4.152**
12 SMB 003	3.612	1.538	**2.575**
12 SMB 028	6.609	11.14	**8.875**
21 DAP 023	2.413	1.637	**2.025**
9 SAB 021	13.522	9.768	**11.645**
12 SMB 011	27.439	24.529	**25.984**
21 DAP 049	7.637	3.191	**5.414**
9 SAB 072	13.426	6.858	**10.142**
9 SAB 076	4.506	1.674	**3.090**
9 SAB 078	4.07	2.973	**3.522**
8 SAB 070	11.736	6.972	**9.354**
21 DAP 046	7.101	5.184	**6.143**
10 SAB 037	5.715	3.351	**4.533**
10 SAB 055	2.735	2.108	**2.422**

10. Raw data for the Alamar Blue® assay

Trypanosoma evansi (STIB 806K):

Standard drugs

Drug	*Alamar Blue Assay for STIB 806K*			
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
				Average
Suramin	88.253	86.181	88.269	**87.568**
Diminazene	13.619	11.85	11.947	**12.472**
Cymelarsan	0.743	1.124	1.369	**1.079**
Quinapyramine	0.134	0.124	0.097	**0.118**

Boykin compounds

Compound	*Alamar Blue Assay for STIB 806K*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
75	3.324	1.245	**2.285**
103	5.192	6.807	**6.000**
201	3.783	5.209	**4.496**
210	0.659	1.757	**1.208**
211	9.215	11.609	**10.412**
217	3.131	1.271	**2.201**
242	7.036	12.585	**9.811**
262	7.186	9.251	**8.219**
320	3.306	6.69	**4.998**
325	1.838	3.729	**2.784**
346	5.089	6.523	**5.806**
351	3.22	3.33	**3.275**
417	3.239	3.965	**3.602**
427	5.788	8.509	**7.149**
484	7.602	5.435	**6.519**
497	9.278	7.732	**8.505**
508	9.871	9.271	**9.571**
544	3.207	4.161	**3.684**
545	8.879	8.299	**8.589**
560	3.82	3.515	**3.668**
609	8.121	8.498	**8.310**
690	5.65	8.884	**7.267**
746	6.345	14.886	**10.616**
763	3.199	2.278	**2.739**
773	4.061	3.423	**3.742**
820	4.596	6.217	**5.407**

Compound	*Alamar Blue Assay for STIB 806K*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
828	4.972	5.676	**5.324**
832	10.254	7.107	**8.681**
841	5.913	8.414	**7.164**
846	9.415	10.565	**9.990**
849	2.432	1.113	**1.773**
850	4.319	4.574	**4.447**
851	4.997	9.763	**7.380**
853	6.483	7.965	**7.224**
866	4.113	2.742	**3.428**
867	1.488	2.003	**1.746**
877	5.635	1.18	**3.408**
902	4.785	2.28	**3.533**
911	3.406	3.978	**3.692**
930	3.211	5.389	**4.300**
935	4.164	4.925	**4.545**
943	2.916	2.682	**2.799**
945	2.713	3.74	**3.227**
988	4.167	7.014	**5.591**
989	9.265	4.968	**7.117**
994	9.719	3.405	**6.562**
1012	8.894	5.557	**7.226**
1016	9.648	11.327	**10.488**
1017	6.21	9.873	**8.042**
1019	2.09	3.598	**2.844**
1023	6.819	6.096	**6.458**
1044	10.221	12.913	**11.567**

Compound	*Alamar Blue Assay for STIB 806K*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
1046	2.532	3.941	**3.237**
1049	6.849	7.188	**7.019**
1050	5.619	6.914	**6.267**
1052	10.65	9.652	**10.151**
1055	12.431	10.342	**11.387**
1065	3.546	1.155	**2.351**
1077	8.132	8.348	**8.240**
1114	1.758	1.597	**1.678**
1149	6.351	5.926	**6.139**
1152	2.549	4.909	**3.729**
1157	3.346	3.698	**3.522**
1159	3.045	4.579	**3.812**
1164	10.77	7.077	**8.924**
1165	3.392	1.377	**2.385**
1171	1.365	1.193	**1.279**
1172	3.378	3.124	**3.251**
1173	5.707	6.592	**6.150**
1177	6.303	3.642	**4.973**
1191	1.787	5.775	**3.781**
1192	10.886	10.03	**10.458**
1194	1.095	1.631	**1.363**
1197	3.214	2.549	**2.882**
1206	11.575	5.743	**8.659**

Compound	*Alamar Blue Assay for STIB 806K*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
1210	1.281	1.29	**1.286**
1213	10.881	8.801	**9.841**
1214	1.008	1.572	**1.290**
1220	7.179	4.572	**5.876**
1228	13.84	9.049	**11.445**
1236	9.919	9.082	**9.501**
1237	8.612	5.08	**6.846**
1239	13.548	12.817	**13.183**
1242	4.148	3.276	**3.712**
1250	5.638	7.062	**6.350**
1253	2.095	4.223	**3.159**
1255	6.42	4.84	**5.630**
1258	7.805	8.878	**8.342**
1265	7.199	9.272	**8.236**
1266	7.376	8.633	**8.005**
1271	8.199	5.837	**7.018**
1272	6.99	4.498	**5.744**
1283	7.954	5.011	**6.483**
1288	5.86	4.859	**5.360**
1340	3.671	3.945	**3.808**
1342	3.633	4.027	**3.830**
1370	5.276	4.899	**5.087**
1371	13.205	10.832	**12.019**

Tidwell compounds

Compound	*Alamar Blue Assay for STIB 806K*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
3 HXC 023	9.565	9.384	**9.475**
5 BGR 066	14.064	15.205	**14.635**
5 BGR 068	7.85	7.133	**7.492**
5 BGR 086	4.245	6.987	**5.616**
5 BGR 088	9.692	10.887	**10.290**
5 BGR 094	24.466	25.007	**24.737**
5 BGR 096	26.714	28.678	**27.696**
6 BGR 012	23.661	25.425	**24.543**
1 SMB 015	9.739	9.763	**9.751**
6 EVK 012	19.324	23.531	**21.428**
1 RRT 039	2.056	3.721	**2.889**

Compound	*Alamar Blue Assay for STIB 806K*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
6 MAA 143	26.805	29.363	**28.084**
5 MAA 085	23.584	21.663	**22.624**
1 KAO 045	15.058	14.748	**14.903**
3 STL 057	4.581	4.133	**4.357**
5 MAA 083	27.71	24.406	**26.058**
5 MAA 101	12.565	9.799	**11.182**
5 MAA 121	19.185	20.578	**19.882**
5 MAA 123	26.819	22.208	**24.514**
5 MAA 137	27.214	25.037	**26.126**
6 MAA 025	1.608	3.189	**2.399**
2 SAB 087	27.622	23.882	**25.752**

Compound	*Alamar Blue Assay for STIB 806K*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
3 SMB 051	9.061	9.352	**9.207**
3 SMB 065	9.648	5.293	**7.471**
3 SMB 079	21.551	20.274	**20.913**
3 SMB 101	6.285	6.494	**6.390**
4 SMB 058	23.056	25.116	**24.086**
4 SMB 092	19.215	18.619	**18.917**
5 SMB 032	5.204	3.641	**4.423**
1 EVK 057	6.457	5.612	**6.035**
16 DAP 022	26.596	23.194	**24.895**
4 SAB 075	2.074	3.162	**2.618**
5 SMB 093	24.813	19.672	**22.243**
16 DAP 095	14.895	16.111	**15.503**
3 KEG 083	6.661	9.788	**8.225**
3 NAN 027	8.377	9.513	**8.945**
6 SAB 038	12.973	10.535	**11.754**
8 SMB 021	3.978	4.348	**4.163**
8 SMB 044	12.774	10.283	**11.529**
7 SAB 004	3.671	3.509	**3.590**
7 SAB 015	10.612	10.026	**10.319**
7 EVK 097	2.047	2.2	**2.124**
7 SAB 079	3.091	3.124	**3.108**
8 EVK 024	2.833	2.558	**2.696**
8 EVK 030	1.19	2.965	**2.078**
19 DAP 021	10.33	11.385	**10.858**
19 DAP 025	1.008	2.655	**1.832**
1 FMS 034	9.302	9.808	**9.555**
1 MCC 112	3.839	1.024	**2.432**
1 MCC 179	4.354	1.454	**2.904**
1 MCC 097	16.801	12.177	**14.489**
1 MCC 128	16.432	10.177	**13.305**
1 MCC 148	2.621	3.836	**3.229**

Compound	*Alamar Blue Assay for STIB 806K*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
1 MCC 184	3.375	5.231	**4.303**
10 SMB 038	18.564	18.907	**18.736**
0 DWB 082	12.527	12.496	**12.512**
0 MXB 736	24.619	26.057	**25.338**
0 MXB 767	14.788	11.496	**13.142**
0 MXB 864	8.979	4.986	**6.983**
10 SMB 084	32.765	39.026	**35.896**
11 SMB 003	12.099	14.661	**13.380**
19 DAP 075	16.505	13.365	**14.935**
19 DAP 085	22.891	21.021	**21.956**
19 DAP 089	7.245	6.089	**6.667**
20 DAP 001	7.86	7.312	**7.586**
19 DAP 079	17.681	17.525	**17.603**
8 SAB 066	5.174	2.541	**3.858**
8 SAB 068	3.487	3.876	**3.682**
8 SAB 072	9.293	6.678	**7.986**
20 DAP 055	9.233	6.472	**7.853**
20 DAP 059	13.79	13.794	**13.792**
12 SMB 003	4.503	6.318	**5.411**
12 SMB 028	3.838	1.084	**2.461**
21 DAP 023	5.646	4.414	**5.030**
9 SAB 021	13.184	13.455	**13.320**
12 SMB 011	30.071	26.946	**28.509**
21 DAP 049	7.568	7.162	**7.365**
9 SAB 072	13.438	9.134	**11.286**
9 SAB 076	10.625	4.535	**7.580**
9 SAB 078	9.408	4.813	**7.111**
8 SAB 070	14.434	12.599	**13.517**
21 DAP 046	15.69	12.409	**14.050**
10 SAB 037	4.482	5.373	**4.928**
10 SAB 055	6.265	3.244	**4.755**

11. Raw data for the Alamar Blue® assay

Trypanosoma brucei brucei (STIB 777S / TbAT1 K.O.):

Standard drugs

Drug	*Alamar Blue Assay for TbAT1 K.O.*			
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
				Average
Suramin	>100	>100	>100	**>100**
Diminazene	>100	>100	>100	**>100**
Cymelarsan	2.917	3.364	3.475	**3.252**
Quinapyramine	>100	81.025	81.043	**>87.356**

Boykin compounds

Compound	*Alamar Blue Assay for TbAT1 K.O.*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
75	14.975	15.787	**15.381**
103	18.326	18.764	**18.545**
201	21.147	20.597	**20.872**
210	18.094	18.416	**18.255**
211	4.312	5.148	**4.730**
217	2.899	4.935	**3.917**
242	8.266	7.496	**7.881**
262	4.005	4.733	**4.369**
320	2.545	3.041	**2.793**
325	2.831	3.461	**3.146**
346	1.187	1.201	**1.194**
351	0.735	0.905	**0.820**
417	3.571	3.627	**3.599**
427	6.148	5.336	**5.742**
484	2.984	3.346	**3.165**
497	5.929	6.037	**5.983**
508	4.892	5.332	**5.112**
544	5.888	6.132	**6.010**
545	11.798	13.196	**12.497**
560	2.349	2.893	**2.621**
609	17.662	19.284	**18.473**
690	1.915	2.035	**1.975**
746	13.807	13.275	**13.541**
763	5.313	6.025	**5.669**
773	84.574	59.184	**71.879**
820	3.108	2.804	**2.956**

Compound	*Alamar Blue Assay for TbAT1 K.O.*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
828	79.232	71.908	**75.570**
832	6.506	5.782	**6.144**
841	77.211	86.137	**81.674**
846	4.796	5.074	**4.935**
849	6.389	6.453	**6.421**
850	8.493	9.941	**9.217**
851	15.526	15.550	**15.538**
853	21.675	27.141	**24.408**
866	23.413	27.865	**25.639**
867	1.527	0.661	**1.094**
877	0.239	0.215	**0.227**
902	31.455	26.327	**28.891**
911	2.768	2.104	**2.436**
930	20.280	18.780	**19.530**
935	69.784	98.170	**83.977**
943	4.316	3.502	**3.909**
945	4.127	3.389	**3.758**
988	63.518	45.394	**54.456**
989	3.471	3.777	**3.624**
994	1.077	1.303	**1.190**
1012	2.498	2.864	**2.681**
1016	8.915	9.451	**9.183**
1017	5.079	4.781	**4.930**
1019	0.512	0.442	**0.477**
1023	7.185	6.003	**6.594**
1044	5.631	4.925	**5.278**

Compound	*Alamar Blue Assay for TbAT1 K.O.*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
1046	>100	87.380	**>93.690**
1049	12.699	18.765	**15.732**
1050	7.927	8.241	**8.087**
1052	7.364	6.864	**7.114**
1055	4.251	2.487	**3.369**
1065	0.136	0.146	**0.141**
1077	55.628	66.830	**61.229**
1114	5.538	5.946	**5.742**
1149	3.861	4.737	**4.299**
1152	0.738	0.624	**0.681**
1157	12.066	11.484	**11.775**
1159	5.827	5.999	**5.913**
1164	0.207	0.161	**0.184**
1165	0.684	1.752	**1.218**
1171	0.397	1.115	**0.756**
1172	3.288	2.594	**2.941**
1173	10.755	11.767	**11.261**
1177	4.293	3.043	**3.668**
1191	9.451	9.501	**9.476**
1192	0.611	0.355	**0.483**
1194	1.399	1.875	**1.637**
1197	15.106	14.598	**14.852**
1206	0.097	0.291	**0.194**

Compound	*Alamar Blue Assay for TbAT1 K.O.*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
1210	2.778	3.596	**3.187**
1213	0.532	0.696	**0.614**
1214	4.193	4.689	**4.441**
1220	13.594	20.268	**16.931**
1228	2.551	3.531	**3.041**
1236	4.762	5.628	**5.195**
1237	9.517	9.001	**9.259**
1239	4.733	4.743	**4.738**
1242	4.867	6.521	**5.694**
1250	0.974	1.662	**1.318**
1253	3.565	5.069	**4.317**
1255	6.187	5.683	**5.935**
1258	8.129	6.813	**7.471**
1265	4.133	4.033	**4.083**
1266	8.879	10.645	**9.762**
1271	9.636	9.866	**9.751**
1272	7.121	6.563	**6.842**
1283	2.842	2.586	**2.714**
1288	0.485	0.921	**0.703**
1340	1.720	0.590	**1.155**
1342	8.396	6.894	**7.645**
1370	0.567	0.429	**0.498**
1371	12.400	11.168	**11.784**

Tidwell compounds

Compound	*Alamar Blue Assay for TbAT1 K.O.*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
3 HXC 023	3.749	4.687	**4.218**
5 BGR 066	5.858	5.532	**5.695**
5 BGR 068	7.415	6.353	**6.884**
5 BGR 086	2.619	2.209	**2.414**
5 BGR 088	4.687	6.317	**5.502**
5 BGR 094	8.069	7.659	**7.864**
5 BGR 096	8.513	8.121	**8.317**
6 BGR 012	3.983	3.407	**3.695**
1 SMB 015	98.561	97.163	**97.862**
6 EVK 012	24.937	16.315	**20.626**
1 RRT 039	28.714	32.682	**30.698**

Compound	*Alamar Blue Assay for TbAT1 K.O.*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
6 MAA 143	>100	>100	**>100**
5 MAA 085	>100	>100	**>100**
1 KAO 045	51.832	59.756	**55.794**
3 STL 057	22.037	15.299	**18.668**
5 MAA 083	>100	>100	**>100**
5 MAA 101	85.668	>100	**>92.834**
5 MAA 121	>100	>100	**>100**
5 MAA 123	98..016	98.342	**98.179**
5 MAA 137	83.177	89.233	**86.205**
6 MAA 025	27.945	26.717	**27.331**
2 SAB 087	79.829	90.905	**85.367**

Compound	*Alamar Blue Assay for TbAT1 K.O.*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
3 SMB 051	>100	>100	**>100**
3 SMB 065	98.613	88.641	**93.627**
3 SMB 079	>100	>100	**>100**
3 SMB 101	66.198	61.390	**63.794**
4 SMB 058	>100	>100	**>100**
4 SMB 092	>100	>100	**>100**
5 SMB 032	87.341	92.963	**90.152**
1 EVK 057	35.825	32.927	**34.376**
16 DAP 022	>100	>100	**>100**
4 SAB 075	25.389	31.549	**28.469**
5 SMB 093	15.106	19.536	**17.321**
16 DAP 095	13.398	11.998	**12.698**
3 KEG 083	39.761	32.849	**36.305**
3 NAN 027	44.182	41.994	**43.088**
6 SAB 038	5.739	6.539	**6.139**
8 SMB 021	0.724	1.020	**0.872**
8 SMB 044	9.396	10.252	**9.824**
7 SAB 004	2.538	2.812	**2.675**
7 SAB 015	5.533	6.193	**5.863**
7 EVK 097	13.165	14.357	**13.761**
7 SAB 079	2.085	1.793	**1.939**
8 EVK 024	38.373	46.283	**42.328**
8 EVK 030	64.045	53.297	**58.671**
19 DAP 021	4.308	3.480	**3.894**
19 DAP 025	1.647	1.987	**1.817**
1 FMS 034	8.863	8.329	**8.596**
1 MCC 112	2.718	2.642	**2.680**
1 MCC 179	0.566	0.914	**0.740**
1 MCC 097	24.591	28.577	**26.584**
1 MCC 128	3.157	2.609	**2.883**
1 MCC 148	1.563	2.021	**1.792**

Compound	*Alamar Blue Assay for TbAT1 K.O.*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
1 MCC 184	3.986	4.644	**4.315**
10 SMB 038	11.720	10.648	**11.184**
0 DWB 082	3.318	2.896	**3.107**
0 MXB 736	20.887	27.669	**24.278**
0 MXB 767	15.814	16.370	**16.092**
0 MXB 864	8.932	9.554	**9.243**
10 SMB 084	49.789	45.379	**47.584**
11 SMB 003	36.527	27.999	**32.263**
19 DAP 075	14.298	9.984	**12.141**
19 DAP 085	23.649	24.311	**23.980**
19 DAP 089	12.384	14.920	**13.652**
20 DAP 001	10.356	8.928	**9.642**
19 DAP 079	10.827	10.611	**10.719**
8 SAB 066	0.984	1.252	**1.118**
8 SAB 068	0.755	1.065	**0.910**
8 SAB 072	4.887	4.139	**4.513**
20 DAP 055	6.277	6.951	**6.614**
20 DAP 059	4.247	3.683	**3.965**
12 SMB 003	2.298	2.876	**2.587**
12 SMB 028	7.068	6.856	**6.962**
21 DAP 023	1.989	2.085	**2.037**
9 SAB 021	11.437	10.351	**10.894**
12 SMB 011	94.338	99.058	**96.698**
21 DAP 049	10.216	9.448	**9.832**
9 SAB 072	13.173	10.979	**12.076**
9 SAB 076	4.583	4.065	**4.324**
9 SAB 078	6.672	7.444	**7.058**
8 SAB 070	17.746	22.082	**19.914**
21 DAP 046	6.898	7.360	**7.129**
10 SAB 037	5.017	4.699	**4.858**
10 SAB 055	1.968	3.750	**2.859**

12. Raw data for the L6 Cytotoxicity assay

Rat skeletal myoblast cells (L6 cells):

Standard drugs

Drug	*Cytotoxicity Assay for L6 cells*			
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)
				Average
Suramin	>90	>90	>90	**>90**
Diminazene	4.619	4.129	3.526	**4.091**
Cymelarsan	>90	>90	>90	**>90**
Quinapyramine	>90	80.886	79.984	**83.623**
Podophylotoxin	0.0079	0.0079	0.0079	**0.0079**

Boykin compounds

Compound	*Cytotoxicity Assay for L6 cells*			
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)
				Average
75	>90	>90	>90	**>90**
103	>90	>90	>90	**>90**
201	>90	>90	>90	**>90**
210	>90	>90	>90	**>90**
211	>90	>90	>90	**>90**
217	>90	>90	>90	**>90**
242	>90	>90	>90	**>90**
262	84.403	82.002	89.438	**85.28**
320	>90	>90	>90	**>90**
325	>90	>90	>90	**>90**
346	4.195	7.394	7.911	**6.50**
351	>90	85.217	>90	**88.41**
417	>90	>90	>90	**>90**
427	>90	88.602	>90	**89.53**
484	>90	>90	>90	**>90**
497	>90	75.658	>90	**85.22**
508	>90	>90	>90	**>90**
544	>90	>90	>90	**>90**
545	6.397	8.195	5.995	**6.86**
560	>90	>90	>90	**>90**
609	>90	>90	>90	**>90**
690	>90	>90	>90	**>90**
746	50.076	54.92	55.478	**53.49**
763	>90	>90	>90	**>90**
773	>90	>90	>90	**>90**
820	>90	>90	>90	**>90**

Compound	*Cytotoxicity Assay for L6 cells*			
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)
				Average
828	>90	>90	>90	**>90**
832	>90	>90	>90	**>90**
841	>90	>90	>90	**>90**
846	>90	>90	>90	**>90**
849	>90	>90	>90	**>90**
850	>90	>90	>90	**>90**
851	>90	>90	>90	**>90**
853	>90	>90	>90	**>90**
866	>90	>90	>90	**>90**
867	>90	>90	>90	**>90**
877	>90	>90	>90	**>90**
902	>90	>90	>90	**>90**
911	>90	>90	>90	**>90**
930	>90	>90	>90	**>90**
935	>90	>90	>90	**>90**
943	86.034	88.171	>90	**88.07**
945	>90	>90	>90	**>90**
988	>90	>90	>90	**>90**
989	>90	>90	>90	**>90**
994	38.994	39.053	35.488	**37.85**
1012	>90	>90	>90	**>90**
1016	>90	>90	>90	**>90**
1017	>90	>90	>90	**>90**
1019	>90	>90	>90	**>90**
1023	>90	>90	>90	**>90**
1044	>90	>90	>90	**>90**

Compound	*Cytotoxicity Assay for L6 cells*			
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml) *Average*
1046	>90	>90	>90	**>90**
1049	>90	>90	>90	**>90**
1050	>90	>90	>90	**>90**
1052	36.898	38.401	49.399	**41.57**
1055	>90	>90	>90	**>90**
1065	>90	>90	>90	**>90**
1077	>90	>90	>90	**>90**
1114	>90	>90	>90	**>90**
1149	>90	>90	>90	**>90**
1152	37.116	30.839	32.518	**33.49**
1157	>90	>90	86.521	**88.84**
1159	>90	>90	>90	**>90**
1164	>90	>90	>90	**>90**
1165	>90	>90	>90	**>90**
1171	69.745	59.859	58.886	**62.83**
1172	>90	>90	>90	**>90**
1173	>90	>90	>90	**>90**
1177	>90	>90	>90	**>90**
1191	>90	>90	>90	**>90**
1192	>90	>90	>90	**>90**
1194	>90	>90	>90	**>90**
1197	>90	>90	>90	**>90**
1206	>90	>90	>90	**>90**

Compound	*Cytotoxicity Assay for L6 cells*			
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml) *Average*
1210	>90	>90	>90	**>90**
1213	>90	>90	>90	**>90**
1214	>90	>90	>90	**>90**
1220	>90	>90	>90	**>90**
1228	>90	>90	>90	**>90**
1236	>90	>90	>90	**>90**
1237	>90	>90	>90	**>90**
1239	>90	>90	>90	**>90**
1242	>90	>90	>90	**>90**
1250	>90	>90	>90	**>90**
1253	>90	>90	>90	**>90**
1255	>90	>90	>90	**>90**
1258	>90	>90	>90	**>90**
1265	>90	>90	>90	**>90**
1266	>90	>90	>90	**>90**
1271	>90	>90	>90	**>90**
1272	>90	>90	>90	**>90**
1283	>90	>90	>90	**>90**
1288	>90	>90	>90	**>90**
1340	>90	>90	>90	**>90**
1342	>90	>90	>90	**>90**
1370	>90	>90	>90	**>90**
1371	>90	>90	>90	**>90**

Tidwell compounds

Compound	*Cytotoxicity Assay for L6 cells*			
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml) *Average*
3 HXC 023	>90	>90	>90	**>90**
5 BGR 066	>90	>90	>90	**>90**
5 BGR 068	>90	>90	>90	**>90**
5 BGR 086	>90	>90	>90	**>90**
5 BGR 088	>90	>90	>90	**>90**
5 BGR 094	>90	>90	>90	**>90**
5 BGR 096	>90	>90	>90	**>90**
6 BGR 012	>90	>90	>90	**>90**
1 SMB 015	>90	>90	>90	**>90**
6 EVK 012	>90	>90	>90	**>90**
1 RRT 039	>90	>90	>90	**>90**

Compound	*Cytotoxicity Assay for L6 cells*			
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml) *Average*
6 MAA 143	>90	>90	>90	**>90**
5 MAA 085	>90	>90	>90	**>90**
1 KAO 045	>90	>90	>90	**>90**
3 STL 057	>90	>90	>90	**>90**
5 MAA 083	>90	>90	>90	**>90**
5 MAA 101	>90	>90	>90	**>90**
5 MAA 121	>90	>90	>90	**>90**
5 MAA 123	>90	>90	>90	**>90**
5 MAA 137	>90	>90	>90	**>90**
6 MAA 025	>90	>90	>90	**>90**
2 SAB 087	>90	>90	>90	**>90**

Compound	*Cytotoxicity Assay for L6 cells*			
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml) *Average*
3 SMB 051	>90	>90	>90	**>90**
3 SMB 065	>90	>90	>90	**>90**
3 SMB 079	>90	>90	>90	**>90**
3 SMB 101	>90	>90	>90	**>90**
4 SMB 058	>90	>90	>90	**>90**
4 SMB 092	>90	>90	>90	**>90**
5 SMB 032	>90	>90	>90	**>90**
1 EVK 057	>90	>90	>90	**>90**
16 DAP 022	>90	>90	>90	**>90**
4 SAB 075	>90	>90	>90	**>90**
5 SMB 093	>90	>90	>90	**>90**
16 DAP 095	37.58	67.4	48.48	**51.15**
3 KEG 083	>90	>90	>90	**>90**
3 NAN 027	>90	>90	>90	**>90**
6 SAB 038	>90	>90	>90	**>90**
8 SMB 021	>90	>90	>90	**>90**
8 SMB 044	>90	>90	>90	**>90**
7 SAB 004	13.99	18.94	34.16	**22.36**
7 SAB 015	>90	>90	>90	**>90**
7 EVK 097	>90	>90	>90	**>90**
7 SAB 079	>90	>90	>90	**>90**
8 EVK 024	>90	>90	>90	**>90**
8 EVK 030	>90	>90	>90	**>90**
19 DAP 021	>90	>90	>90	**>90**
19 DAP 025	>90	>90	>90	**>90**
1 FMS 034	>90	>90	>90	**>90**
8 EVK 024	>90	>90	>90	**>90**
8 EVK 030	>90	>90	>90	**>90**
19 DAP 021	>90	>90	>90	**>90**
19 DAP 025	>90	>90	>90	**>90**
1 FMS 034	>90	>90	>90	**>90**
1 MCC 112	>90	>90	>90	**>90**
1 MCC 179	>90	>90	>90	**>90**
1 MCC 097	>90	>90	>90	**>90**
1 MCC 128	>90	>90	>90	**>90**
1 MCC 148	>90	>90	>90	**>90**

Compound	*Cytotoxicity Assay for L6 cells*			
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml)	IC_{50} (µg/ml) *Average*
1 MCC 184	>90	>90	>90	**>90**
10 SMB 038	>90	>90	>90	**>90**
0 DWB 082	22.51	28.12	23.58	**24.74**
0 MXB 736	>90	>90	>90	**>90**
0 MXB 767	31.65	33.71	36.08	**33.81**
0 MXB 864	>90	>90	>90	**>90**
10 SMB 084	>90	>90	>90	**>90**
11 SMB 003	>90	>90	>90	**>90**
19 DAP 075	>90	>90	>90	**>90**
19 DAP 085	>90	>90	>90	**>90**
19 DAP 089	>90	>90	>90	**>90**
20 DAP 001	>90	>90	>90	**>90**
19 DAP 079	>90	>90	>90	**>90**
8 SAB 066	>90	>90	>90	**>90**
8 SAB 068	>90	>90	>90	**>90**
8 SAB 072	>90	>90	>90	**>90**
20 DAP 055	>90	>90	>90	**>90**
20 DAP 059	>90	>90	>90	**>90**
12 SMB 003	>90	>90	>90	**>90**
12 SMB 028	>90	>90	>90	**>90**
21 DAP 023	>90	>90	>90	**>90**
9 SAB 021	>90	>90	>90	**>90**
12 SMB 011	>90	>90	>90	**>90**
21 DAP 049	>90	>90	>90	**>90**
9 SAB 072	>90	>90	>90	**>90**
9 SAB 076	>90	>90	>90	**>90**
9 SAB 021	>90	>90	>90	**>90**
12 SMB 011	>90	>90	>90	**>90**
21 DAP 049	>90	>90	>90	**>90**
9 SAB 072	>90	>90	>90	**>90**
9 SAB 076	>90	>90	>90	**>90**
9 SAB 078	>90	>90	>90	**>90**
8 SAB 070	>90	>90	>90	**>90**
21 DAP 046	>90	>90	>90	**>90**
10 SAB 037	>90	>90	>90	**>90**
10 SAB 055	>90	>90	>90	**>90**

13. Raw data for strain sensitivity with the Alamar Blue® assay

Trypanosoma evansi (CAN86 / Brazil):

Compound	*Alamar Blue Assay for CAN86/Brazil*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
Suramin	45.682	42.380	**44.031**
Diminazene	11.758	12.600	**12.179**
Cymelarsan	0.892	1.234	**1.063**
Quinapyramine	0.118	0.180	**0.149**
DB 75	2.937	2.225	**2.581**
DB 820	4.595	4.759	**4.677**
DB 867	3.146	2.652	**2.899**
DB 930	3.647	4.719	**4.183**
DB 1192	10.211	8.907	**9.559**
DB 1283	6.084	5.750	**5.917**

Trypanosoma evansi (Colombia):

Compound	*Alamar Blue Assay for Colombia*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
Suramin	61.382	58.188	**59.785**
Diminazene	17.755	14.769	**13.262**
Cymelarsan	1.473	0.905	**1.189**
Quinapyramine	0.157	0.113	**0.135**
DB 75	4.146	3.334	**3.740**
DB 820	3.872	4.278	**4.075**
DB 867	2.311	1.425	**1.868**
DB 930	6.631	6.447	**6.539**
DB 1192	6.734	9.612	**8.173**
DB 1283	5.192	5.958	**5.575**

Trypanosoma evansi (Kazakhstan):

Compound	*Alamar Blue Assay for Kazakhstan*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
Suramin	59.716	62.034	**60.875**
Diminazene	13.187	13.051	**13.119**
Cymelarsan	0.966	1.440	**1.203**
Quinapyramine	0.073	0.105	**0.089**
DB 75	2.279	2.643	**2.461**
DB 820	5.916	5.348	**5.632**
DB 867	1.815	2.765	**2.290**
DB 930	5.341	6.025	**5.683**
DB 1192	10.562	10.882	**10.722**
DB 1283	5.938	6.620	**6.279**

Trypanosoma evansi (Philippines):

Compound	*Alamar Blue Assay for Philippines*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
Suramin	50.741	57.003	**53.872**
Diminazene	11.763	12.509	**12.136**
Cymelarsan	1.328	1.224	**1.276**
Quinapyramine	0.084	0.144	**0.114**
DB 75	1.667	0.897	**1.282**
DB 820	2.953	3.747	**3.350**
DB 867	1.064	1.546	**1.305**
DB 930	11.491	9.881	**10.686**
DB 1192	9.833	11.011	**10.422**
DB 1283	9.174	8.114	**8.644**

Trypanosoma evansi (RoTat 1.2):

Compound	*Alamar Blue Assay for RoTat 1.2*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
Suramin	79.891	72.539	**76.215**
Diminazene	12.194	12.968	**12.581**
Cymelarsan	0.989	1.549	**1.269**
Quinapyramine	0.167	0.119	**0.143**
DB 75	2.581	2.735	**2.658**
DB 820	5.210	4.024	**4.617**
DB 867	1.437	1.613	**1.525**
DB 930	3.825	4.365	**4.095**
DB 1192	9.961	9.807	**9.884**
DB 1283	6.198	6.784	**6.491**

Trypanosoma evansi (Vietnam):

Compound	*Alamar Blue Assay for Vietnam*		
	IC_{50} (ng/ml)	IC_{50} (ng/ml)	IC_{50} (ng/ml)
			Average
Suramin	81.638	75.106	**78.372**
Diminazene	12.751	12.287	**12.519**
Cymelarsan	0.963	1.405	**1.184**
Quinapyramine	0.191	0.139	**0.165**
DB 75	2.645	2.427	**2.536**
DB 820	3.975	4.159	**4.067**
DB 867	1.568	1.358	**1.463**
DB 930	3.715	4.025	**3.870**
DB 1192	10.094	10.552	**10.323**
DB 1283	5.581	6.307	**5.944**

14. SOP: *In vivo* acute efficacy test (mouse model)

Standard parasite strains: None

Standard conditions:

Mice: NMRI female mice, 22 - 25 g

Cages: Macrolon, type II cages

Maintenance: 22 °C and 60 - 70 % relative humidity
Pelleted food and water *ad libitum*

Test procedure:

Day 0: All mice in each group (four mice per group), including the control group, are infected with a parasite concentration of $1x10^4$, with the parasite strain being tested. Infection is given using an i.p. route, with each mouse receiving 0.25 ml.

Day 3-6: The mice are then treated over four consecutive days, starting on day 3 post infection and once a parasitaemia has been established. The treatment is also administered using an i.p. route.

Day 7: The parasitaemia for all the mice are checked using a tail blood examination technique. Thereafter, the parasitaemia is monitored twice a week, using the tail blood examination technique.

Day 60: Any surviving and aparasitaemic mice are considered cured and thus are euthanized on the final day of the experiment.

15. Example record sheet for the *in vivo* efficacy test (mouse model)

D = Died T = Treatment

NMRI females		Date :	02.05	03.05	04.05	05.05	06.05	11.05								
inf. : 29.04.2005	T 0	Days	3	4	5	6	7	12								
	K						*D*									
Control	R					*D*										
	St					*D*										
1	KSt							*D*								

Average survival (in days) = **7.75**

NMRI females		Date :	02.05	03.05	04.05	05.05	06.05	10.05	17.05	23.05	27.05	30.05	07.06	13.06	21.06	28.06
inf. : 29.04.2005	T 0	Days	3	4	5	6	7	11	18	24	28	31	39	45	53	60
	K		T	T	T	T	-	-	-	-	-	-	-	-	-	-
DB 75	R		T	T	T	T	-	-	-	-	-	-	-	-	-	-
(0.5mg/kg)	St		T	T	T	T	-	-	-	-	-	-	-	-	-	-
2	KSt		T	T	T	T	-	-	-	-	-	-	-	-	-	-

Average survival (in days) = **>60**

16. Raw data for the *in vivo* dose-response efficacy test (mouse model)

Trypanosoma evansi (STIB 806K):

Lowest curative doses are highlighted in green

Compound Identity	Dose tested (x4) (mg/kg mouse)	% survival	Average (days)
Standard drugs			
Suramin	1	100	>60
	0.5	75	>51
	0.25	0	10.75
Diminazene	1	75	>43.75
	0.2	0	8.5
Cymelarsan	1	100	>60
	0.25	100	>60
	0.2	100	>60
	0.125	100	>60
	0.0625	100	>60
	0.03125	25	>36.75
Quinapyramine	1	100	>60
	0.5	75	>56.75
	0.25	25	>41
DB 75	1	100	>60
	0.5	100	>60
	0.25	100	>60
	0.2	100	>60
	0.125	75	>55
	0.0625	25	>28.50
Boykin			
DB 211	0.5	0	15
DB 320	1	75	>49.25
DB 690	1	100	>60
	0.5	75	>56.25
DB 820	1	100	>60
	0.25	100	>60
	0.2	75	>45.25
	0.125	50	>50.25
DB 828	1	0	23.25
DB 850	0.5	0	15.5
DB 853	1	0	18.25
DB 867	1	100	>60
	0.5	100	>60
	0.25	75	>53.50
	0.125	50	>46.00

Lowest curative doses are highlighted in green

Compound Identity	Dose tested (x4) (mg/kg mouse)	% survival	Average (days)
DB 877	1	25	>35
	0.2	0	12.25
DB 930	1	100	>60
	0.5	100	>60
	0.25	25	>37.50
	0.125	25	>32.25
DB 989	0.5	0	26.75
DB 1012	1	0	12.5
	0.2	0	8
DB 1017	0.5	25	>42.50
DB 1019	1	25	>28.25
DB 1044	0.5	0	16.25
DB 1046	1	0	10.75
DB 1052	1	75	>52.75
DB 1055	0.5	0	11.5
DB 1152	1	0	20.25
	0.2	0	14.25
DB 1171	0.5	25	>30.25
DB 1172	1	25	>38.75
DB 1173	0.5	0	14.5
DB 1177	0.5	0	12.5
DB 1191	0.5	0	6.75
DB 1192	0.5	100	>60
	0.25	50	>50.75
	0.125	25	>28.50
DB 1194	0.5	0	15.75
DB 1213	0.5	0	26.75
DB 1220	0.5	0	12.75
DB 1237	0.5	50	>46.50
	0.25	0	16
DB 1253	0.5	0	6.75
DB 1258	0.5	0	6.75
DB 1272	0.5	0	18
DB 1283	1	100	>60
	0.5	100	>60
	0.25	50	>45.50
	0.125	0	22.5
DB 1288	0.5	0	22.5
DB 1340	0.5	0	7.5

Compound Identity	Dose tested (x4) (mg/kg mouse)	% survival	Average (days)
DB 1342	0.5	25	>33.50
DB 1370	0.5	0	20.75
DB 1371	0.5	0	14.25
Tidwell			
3 KEG 083	1	0	29.25
4 SAB 075	1	50	>43.25
	0.5	0	19.25
5 MAA 101	1	25	>29
5 SMB 093	0.5	0	20.25
8 SAB 066	0.5	50	>38
9 SAB 021	0.5	0	8.5
12 SMB 003	0.5	0	7.75
16 DAP 095	1	100	>60
	0.5	25	>29.25
	0.25	25	>30.75
19 DAP 021	0.5	0	11.75
19 DAP 025	1	100	>60
	0.5	75	>56.25
	0.25	25	>36

Lowest curative doses are highlighted in green

17. <u>Protocol:</u> Preliminary Toxicity Study for DB 75, DB 867 and DB 1192 in goats in the Canary Islands.

Materials:

- Blood collection tubes (vacutainer system - 2 ml (100 pieces) and 5 ml (10 pieces)
- Eppendorf tubes (for plasma transfer)
- Cryotubes (for plasma storage)
- Pipettes (1000 µl and 100 µl)
- Corresponding tips for pipettes
- Centrifuge

Experimental procedure:

1) Six female Canary goats, weighing between 25 – 30 kg and no less than 6 months old are purchased from a commercial farmer and transported to the faculty. Once there, they are housed inside enclosed pens with food and water *ad libitum*. The goats are left for a week to acclimatise to the new surroundings.

2) At the start of the toxicity experiment, the goats are divided into three groups of two goats. Each group is allocated to one of the three lead compounds as shown in the table.

Group Number	Number of goats	Lead compound	Colour on timetable chart
1	2	DB 75	Green
2	2	DB 867	Purple
3	2	DB 1192	Yellow

3) On day **1** of the experiment, a **3 ml** blood sample is taken from all **6** goats to determine control plasma levels. Each blood sample is inverted **8-10** times to enable thorough mixing with lithium heparin (or EDTA) (sprayed on the internal tube walls) to prevent coagulation.

4) Once fully mixed, the tubes are placed into a centrifuge and spun at **3000 *g*** for **10 minutes** to separate the blood cells from the plasma. After centrifugation, the plasma is removed and placed into a sterile polypropylene cryotube and stored away from light at -25 °C. The remaining blood is discarded.

5) On day **2**, one goat is taken from group **1** (corresponding to **DB 75**) and a **2 ml** blood sample is taken from this goat. This blood sample is also inverted **8-10** times, centrifuged at **3000 *g*** for **10 minutes** and the plasma removed, placed into a polypropylene cryotube and stored away from light at -25 °C.

6) Half an hour later, a **1 ml / 10 kg** single compound injection of a **40 mg/ml** dose of **DB 75** is given to this goat. The compound is administered using an intramuscular route. Further **2 ml** blood samples are taken from the same goat at specific time intervals of **15** minutes, **30** minutes, **1** hour, **2** hours, **4** hours, **8** hours, **24** hours and **48** hours post-injection.

7) These **eight** blood samples are handled in the exact same way as the blood sample in step **5**.

8) On day **4**, once the final **2ml** blood sample has been taken (**48** hours), both goats in group **1** (corresponding to **DB 75**) are given a **1 ml / 10 kg** compound injection of a **10 mg/ml** dose of **DB 75**. Again the compound is administered using an intramuscular route.

9) Approximately five minutes BEFORE a second application of a **1 ml / 10 kg** compound injection of a **10 mg/ml** dose of **DB 75** is given to both goats, **2** hours after the **first** application was given, a **2 ml** blood sample is taken from both goats in group **1**. These blood samples are taken to determine the trough level of the compound within the goats, just before the next compound application is administered. These blood samples are treated exactly the same as explained in step **5**.

10) Similarly, approximately five minutes BEFORE a third application of a **1 ml / 10 kg** compound injection of a **10 mg/ml** dose of **DB 75** is given to both goats in group **1**, **2** hours after the **second** application was given, a **2 ml** blood sample is taken from these two goats to again determine the trough levels. And similarly to step **9**, these blood samples are handled in the same way as seen in step **5**.

11) Approximately five minutes BEFORE a fourth (and final) application of a **1 ml / 10 kg** compound injection of a **10 mg/ml** dose of **DB 75** is given to both goats, **2** hours after the **third** application was given, a **2 ml** blood sample is taken from these two goats to determine the final trough level. Again the blood samples are handled as described in step **5**.

12) In total, both goats in group **1** have now received an accumulated compound dose of **4 mg/kg** of **DB 75** over a **6** hour time period.

13) Further **2 ml** blood samples are taken from the same two goats in group **1** at specific time intervals of **15** minutes, **30** minutes, **1** hour, **2** hours, **4** hours, **8** hours, **24** hours, **48** hours, **72** hours and **96** hours after the final (**fourth**) **1 mg/kg** compound application. The blood samples are handled as shown in step **5**.

14) The procedure steps **5** to **13** are then repeated using the goats in group **2** corresponding with **DB 867** and then with the goats in group **3** corresponding with **DB 1192**.

15) The experiment for group **2** (corresponding with **DB 867**) starts on day **3**, whereas the experiment for group **3** (corresponding with **DB 1192**) begins on day **8**.

16) The allocated timing involved in the three toxicity experiments can be visualised in the timetables for week **1** (days **1 - 7**) and week **2** (days **8 - 14**). The three different lead compounds (**DB 75**, **DB 867** and **DB 1192**) are assigned three different colours (**green**, **purple** and **yellow**, respectively) to aid visualisation of the sampling and compound administration protocols.

17) During all three toxicity experiments, all goats must be observed for signs of **acute toxicity**, which include tremors, extreme salivation, lacrimation/tearing, abnormal urination, rapid onset of diarrhoea, hypotension and possible hypertension.

Week 1	**Monday** (day 1)	**Tuesday** (day 2)	**Wednesday** (day 3)	**Thursday** (day 4)	**Friday** (day 5)	**Saturday** (day 6)	**Sunday** (day 7)
08.30							
09.00	Take control plasma from **all 6** goats (2ml vol.)	Take 2ml blood sample from goat 1 for **DB 75**	**09.40** = 2ml blood sample (goat 1-**24hr**)	**09.24** = 2ml blood sample (goat 1-**24**hr)	**09.24** = 2ml blood sample (goat 1-**48**hr)		
09.30		Give single **4mg/kg** injection **DB75** (goat 1 only)	Take 2ml sample from goat 1 for **DB867** **09.24** = Give single **4mg/kg** injection **DB867** (goat 1 only) **09.39 + 09.54** = 2ml blood samples (goat **1**) **10.24** = 2ml blood sample (goat 1-**1**hr)	**09.40** = 2ml blood sample (goat 1-**48hr**)	**09.25 + 09.28** = Give **1**st injection (**1mg/kg**) **2** goats		
10.00		**09.55 + 10.10** = 2ml blood samples (goat 1)		**09.50 + 09.55** = Give **1**st injection (**1mg/kg**) **2** goats			
10.30		**10.40** = 2ml blood sample (goat 1-**1hr**)					
11.00					**11.18 + 11.22** = 2ml blood sample (trough) **2** goats		
11.30		**11.40** = 2ml blood sample (goat 1-**2hr**)	**11.24** = 2ml blood sample (goat 1-**2**hr)	**11.48 + 11.49** = 2ml blood sample (trough) **2** goats.	**11.20 + 11.24** = Give **2**nd injection (**1mg/kg**) **2** goats		
12.00				**11.50 + 11.55** = Give **2**nd injection (**1mg/kg**) **2** goats			
12.30							
13.00			**13.24** = 2ml blood sample (goat 1-**4**hr)		**13.23 + 13.26** = 2ml blood sample (trough) **2** goats		

13.30		**13.40** = 2ml blood sample (goat 1-**4hr**)		**13.42** + **13.44** = 2ml blood sample (trough) **2** goats.	**13.25** + **13.28** = Give **3rd** injection (**1mg/kg**) **2** goats		
14.00				**13.45** + **13.50** = Give **3rd** injection (**1mg/kg**) **2** goats			
14.30							
15.00					**15.23** + **15.26** = 2ml blood sample (trough) **2** goats		
15.30					**15.25** + **15.28** = Give **4th** injection (**1mg/kg**) **2** goats	**15.25** + **15.28** = 2ml blood samples (**2** goats-**24**hr)	**15.25** + **15.28** = 2ml blood samples (**2** goats-
16.00				**16.05** + **16.10** = 2ml blood sample (trough) **2** goats.	**15.40** + **15.43** = 2ml blood samples (**2** goats) **15.55** + **15.58** = 1ml blood samples (**2** goats)	**16.15** + **16.20** = 2ml blood samples (**2** goats-**48**hr)	**16.15** + **16.20** = 2ml blood samples (**2** goats-
16.30				**16.15** + **16.20** = Give **4th** injection (**1mg/kg**) **2** goats			
17.00			**17.24** = 2ml blood sample (goat 1-**8**hr)	**16.30** + **16.35** = 2ml blood samples (**2** goats) **16.45** + **16.50** = 2ml blood samples (**2** goats)	**16.15** + **16.20** = 2ml blood samples (**2** goats-**24**hr)		
17.30		**17.40** = 2ml blood sample (goat 1-**8hr**)		**17.15** + **17.20** =2ml blood samples (**2** goats-**1**hr)	**16.25** + **16.28** = 2ml blood samples (**2** goats-**1**hr)		
18.00					**17.25** + **17.28** = 2ml blood samples (**2** goats-**2**hr)		
18.30				**18.15** + **18.20** = 2ml blood samples (**2** goats-**2**hr)			

19.00							
19.30					**19.25 + 19.28** = 2ml blood samples (**2** goats-**4**hr)		
20.00				**20.15 + 20.20** = 2ml blood samples (**2** goats-**4**hr)			
20.30							
21.00							
21.30							
22.00							
22.30							
23.00					**23.25 + 23.28** = 2ml blood samples (**2** goats-**8**hr)		
23.30				**23.30 + 23.35** = 2ml blood samples (**2** goats-**8**hr)			
00.00							

Week 2	**Monday** (day 8)	**Tuesday** (day 9)	**Wednesday** (day 10)	**Thursday** (day 11)	**Friday** (day 12)	**Saturday** (day 13)	**Sunday** (day 14)
08.30	Take 2ml blood sample from goat 1 for **DB1192**	**07.45** = 2ml blood sample (goat 1-**24hr**)	**07.45** = 2ml blood sample (goat 1-**48hr**)				
09.00	Give single **4mg/kg** injection **DB1192** (goat 1 only) **08.00 + 08.15** = 2ml blood samples (goat 1) **08.45** = 2ml blood sample (goat 1-**1hr**) **09.45** = 2ml blood sample (goat 1-**2hr**) **11.45** = 2ml blood sample (goat 1-**4hr**)		**07.50 + 07.55** = Give **1st** injection (**1mg/kg**) **2** goats				
09.30			**09.48 + 09.53** = 2ml blood sample (trough) **2** goats.				**09.50 + 09.55** = 2ml blood samples (**2** goats-
10.00			**09.50 + 09.55** = Give **2nd** injection (**1mg/kg**) **2** goats				
10.30							
11.00			**11.48 + 11.53** = 2ml blood sample (trough) **2** goats.				
11.30			**11.50 + 11.55** = Give **3rd** injection (**1mg/kg**) **2** goats				
12.00							
12.30							
13.00			**13.48 + 13.53** = 2ml blood sample (trough) **2** goats				

13.30			**13.50 + 13.55** = Give **4th** injection (**1mg/kg**) **2** goats	**13.50 + 13.55** = 2ml blood samples (**2** goats-**24**hr)	**13.50 + 13.55** = 2ml blood samples (**2** goats-	**13.50 + 13.55** = 2ml blood samples (**2** goats-**72**hr)	
14.00			**14.05 + 14.10** = 2ml blood samples (**2** goats) **14.20 + 14.25** = 2ml blood samples (**2** goats)				
14.30							
15.00	**15.25 + 15.28** = 2ml blood samples (**2** goats-**72**hr)	**15.25 + 15.28** = 2ml blood samples (**2** goats-**96**hr)	**14.50 + 14.55** = 2ml blood samples (**2** goats-**1**hr)				
15.30	**15.45** = 2ml blood sample (goat 1-**8hr**)		**15.50 + 15.55** = 2ml blood samples (**2** goats-**2**hr)				
16.00	**16.15 + 16.20** = 2ml blood samples (**2** goats-**96**hr)						
16.30							
17.00							
17.30			**17.50 + 17.55** = 2ml blood samples (**2** goats-**4**hr)				
18.00							
18.30							

19.00							
19.30							
20.00							
20.30							
21.00							
21.30			**21.50 + 21.55** = 2ml blood samples (**2** goats-**8**hr)				
22.00							
22.30							
23.00							
23.30							
00.00							

18. Raw data for the *in vivo* preliminary toxicity test (goat model)

DB 75 (4 mg/kg): Goat **0823**

Sample	Time (hrs)	Concentration (ng/ml)
0	0.00	No peak
15 mins	0.25	3690
30 mins	0.50	2910
1 hr	1.00	3290
2 hrs	2.00	3560
4 hrs	4.00	3290
8 hrs	8.00	3680
24 hrs	24.00	3320
48 hrs	48.00	3080

DB 75 (4 x 1 mg/kg): Goat **0865**

Sample	Time (hrs)	Concentration (ng/ml)
1st trough	1.97	1030
2nd trough	3.97	1530
3rd trough	5.97	1790
15 mins	6.25	1830
30 mins	6.50	1790
1 hr	7.00	1810
2 hrs	8.00	1570
4 hrs	10.00	1610
8 hrs	14.00	1510
24 hrs	30.00	1590
48 hrs	54.00	1390
72 hrs	78.00	1490
96 hrs	102.00	1060

DB 867 (4 mg/kg): Goat **0866**

Sample	Time (hrs)	Concentration (ng/ml)
0	0.00	No peak
15 mins	0.25	2260
30 mins	0.50	2880
1 hr	1.00	1850
2 hrs	2.00	2420
4 hrs	4.00	2390
8 hrs	8.00	1930
24 hrs	24.00	2580
48 hrs	48.00	2510

DB 867 (4 x 1 mg/kg): Goat **0850**

Sample	Time (hrs)	Concentration (ng/ml)
1st trough	1.97	3360
2nd trough	3.97	3090
3rd trough	5.97	3070
15 mins	6.25	2520
30 mins	6.50	2970
1 hr	7.00	3390
2 hrs	8.00	3210
4 hrs	10.00	3010
8 hrs	14.00	3010
24 hrs	30.00	3330
48 hrs	54.00	3340
72 hrs	78.00	3090
96 hrs	102.00	2680

DB 1192 (4 mg/kg): Goat **0818**

Sample	Time (hrs)	Concentration (ng/ml)
0	0.00	No peak
15 mins	0.25	1980
30 mins	0.50	1840
1 hr	1.00	774
2 hrs	2.00	446
4 hrs	4.00	561
8 hrs	8.00	346
24 hrs	24.00	140
48 hrs	48.00	193

DB 1192 (4 x 1 mg/kg): Goat **Grey**

Sample	Time (hrs)	Concentration (ng/ml)
1st trough	1.97	265
2nd trough	3.97	338
3rd trough	5.97	560
15 mins	6.25	585
30 mins	6.50	762
1 hr	7.00	631
2 hrs	8.00	570
4 hrs	10.00	475
8 hrs	14.00	352
24 hrs	30.00	59.1
48 hrs	54.00	51.4
72 hrs	78.00	28.7
96 hrs	102.00	29.7

19. <u>Protocol:</u> "Proof of Concept" Trial for DB 75, DB 867 and DB 1192 in goats in the Canary Islands.

Experimental procedure:

18) Twenty-five female Canary goats, weighing between 20 – 52 kg and no less than 6 months old are purchased from a commercial farmer and transported to the faculty. Once there, they are housed inside enclosed fly-proof pens with food and water *ad libitum*. The goats are left for a week to acclimatise to the new surroundings.

19) At the start of the efficacy trial, the goats are divided into seven groups of three goats with four goats in the control group. Each group is allocated to a specific compound and treatment schedule as shown in the table below.

Group Number	Number of goats	Test compound	Treatment schedule
1	4	None	None
2	3	Diminazene	5 mg/kg
3	3	DB 75	2.5 mg/kg
4	3	DB 867	2.5 mg/kg
5	3	DB 1192	2.5 mg/kg
6	3	DB 75	1.25 mg/kg
7	3	DB 867	1.25 mg/kg
8	3	DB 1192	1.25 mg/kg

20) On day **1** of the experiment, a **4 ml** blood sample is taken from all **25** goats to determine that the goats are not infected.

21) Once the blood samples have been collected, the blood is tested using several detection techniques. These include:

    a. Microscopic/direct method = Haematocrit technique (Woo)
    b. Serological/Antibody method = CATT/*T. evansi* technique
    c. DNA detection method = PCR/*T. evansi* technique

22) Once all goats have been successfully checked for no previous *T. evansi* infection, all **25** goats can be infected using *T. evansi* infected blood isolated from a highly parasitaemic camel found positive for *T. evansi* infection.

23) The goats are infected with a **10^6 / ml** parasite concentration, using an **intravenous** route of application.

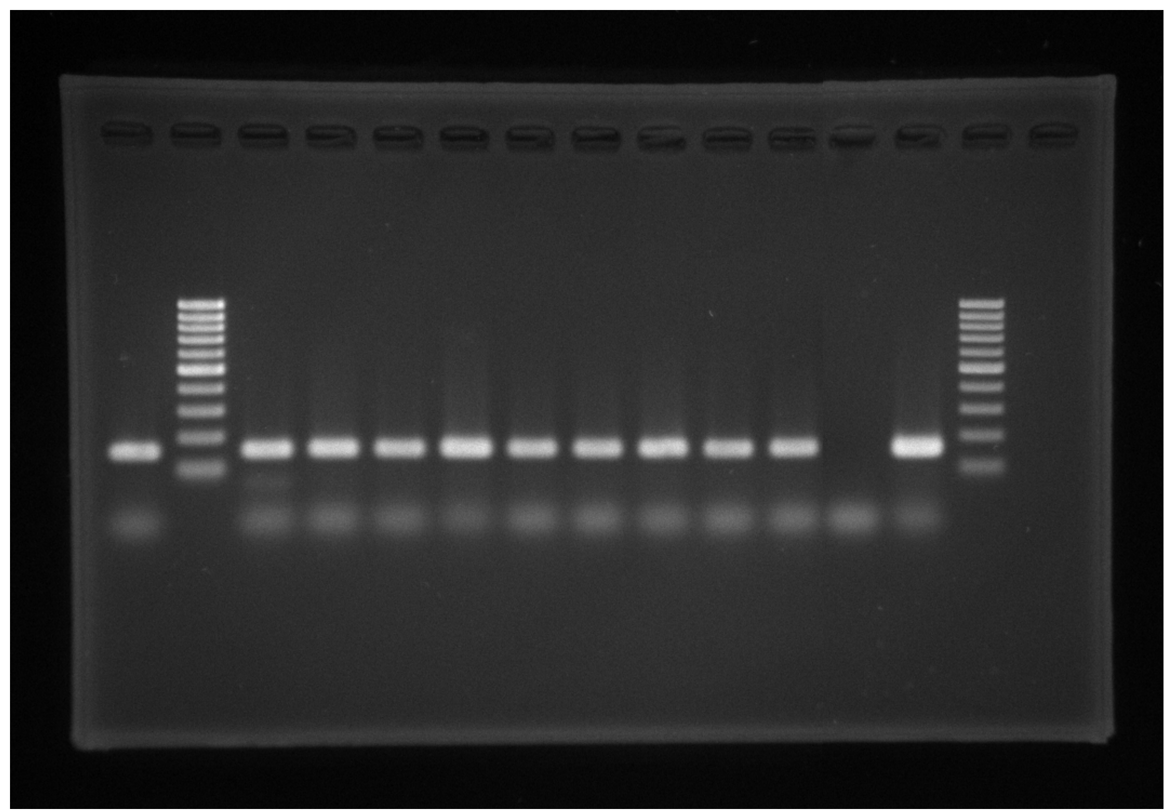
24) A previous blood sample taken from the positive camel will be used to count and calculate the respective concentration of the trypanosomes within the camel blood. Thereafter, the trypanosome suspension will be calculated as required and administered to the goats at a total volume of **1 ml / goat**.

25) The goats are then regularly checked for the presence of parasitaemia within the peripheral blood, using **all three** detection methods.

26) As soon as all groups of goats become positive for *T. evansi* infection, treatment will commence (approximately one month post infection).
27) Treatment will be given using an **intramuscular** route of four applications, spread over eight days for the corresponding scheduled compound dose depending on the group number.
28) After treatment, the goats are checked regularly over the next five months, to determine the presence/absence of *T. evansi* infection and hence the efficacy of the test compounds used in this trial.

20. Agarose gels of goat samples for the PCR / *T. evansi* method

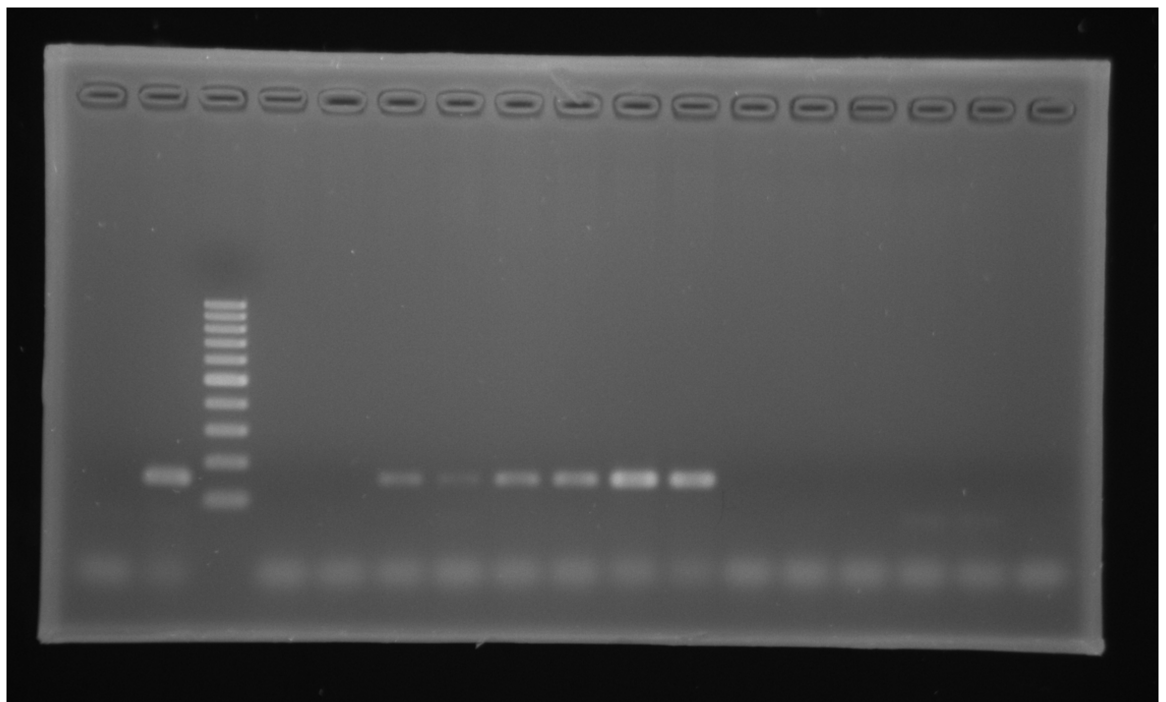
After experimental infection with *T. evansi*, yet before compound treatment:



Lane 1. **1773** (Diminazene)
Lane 2. Ladder
Lane 3. **7396** (Diminazene)
Lane 4. **7388** (Diminazene)
Lane 5. **7372** (DB 867)
Lane 6. **7341** (DB 867)
Lane 7. **7339** (DB 867)
Lane 8. **7319** (DB 867)
Lane 9. **7390** (BD 75)
Lane 10. **7324** (DB 75)
Lane 11. **5406** (DB 75)
Lane 12. Negative control
Lane 13. Positive control
Lane 14. Ladder

All lanes show a positive reaction for parasite DNA except for the ladder lanes (2 and 14) and the negative control (lane 12).

After experimental infection with *T. evansi* and two weeks after compound treatment:



Lane 1. Negative control
Lane 2. Positive control
Lane 3. Ladder
Lane 4. **7341** (DB 867)
Lane 5. **7372** (DB 867)
Lane 6. **7379** (DB 1192)
Lane 7. **7387** (DB 1192)
Lane 8. **2255** (Control)
Lane 9. **7397** (Control)
Lane 10. **7374** (Control)
Lane 11. **7314** (Control)
Lane 12. **1773** (Diminazene)
Lane 13. **7396** (Diminazene)
Lane 14. **7388** (Diminazene)
Lane 15. **7390** (DB 75)
Lane 16. **7324** (DB 75)
Lane 17. **5406** (DB 75)

Lanes 2, 6, 7, 8, 9, 10 and 11 are positive for parasite DNA.

21. <u>Protocol:</u> Pharmacokinetic trial for DB 75, DB 867 and DB 1192 in goats in the Canary Islands.

Experimental procedure:

29) Twenty-five female Canary goats, weighing between 20 – 52 kg and no less than 6 months old are purchased from a commercial farmer and transported to the faculty. Once there, they are housed inside enclosed fly-proof pens with food and water *ad libitum*. The goats are left for a week to acclimatise to the new surroundings.

30) At the start of the pharmacokinetic trial, the goats are divided into seven groups of three goats with four goats in the control group. Each group is allocated to a specific compound and treatment schedule as shown in the table below.

Group Number	Number of goats	Test compound	Treatment schedule
1	4	None	None
2	3	Diminazene	5 mg/kg
3	3	DB 75	2.5 mg/kg
4	3	DB 867	2.5 mg/kg
5	3	DB 1192	2.5 mg/kg
6	3	DB 75	1.25 mg/kg
7	3	DB 867	1.25 mg/kg
8	3	DB 1192	1.25 mg/kg

31) On day **1** of the experiment, a **4 ml** blood sample is taken from all **25** goats to determine that the goats are not infected. The sample is centrifuged at 3000 *g* for ten minutes, the plasma removed and placed into plastic cryotubes and then stored frozen at -80 °C.

32) Once all goats have been successfully checked for no previous *T. evansi* infection, all **25** goats can be infected using *T. evansi* infected blood isolated from a highly parasitaemic camel found positive for *T. evansi* infection.

33) The goats are infected with a **10^6 / ml** parasite concentration, using an **intravenous** route of application.

34) A previous blood sample taken from the positive camel will be used to count and calculate the respective concentration of the trypanosomes within the camel blood. Thereafter, the trypanosome suspension will be calculated as required and administered to the goats at a total volume of **1 ml / goat**.

35) As soon as all groups of goats become positive for *T. evansi* infection, compound treatment will commence (approximately one month post infection).

36) Treatment will be given using an **intramuscular** route of four applications, spread over eight days for the corresponding scheduled compound dose depending on the group number.

37) Blood samples will be collected from each of the goats, shortly before the next compound injection is applied. This will provide data on the trough levels for each compound tested for each goat.

38) After the fourth and final compound injection has been applied, blood samples will be taken from each goat at the following time points: **0.5**, **1**, **2**, **4**, **8**, **24**, **48**, **96** and **192** hours post-treatment.

39) All blood samples taken will be immediately placed on ice and thereafter transported to the laboratory for plasma preparation. The samples will be centrifuged at **3000** *g* for ten minutes, the plasma removed and placed into plastic cryotubes for storage at **-80** °C.

40) Thereafter, the plasma samples will be shipped to the University of North Carolina, Chapel Hill, USA, where they will be analysed using **HPLC** and **MS** techniques to determine the concentration of compound present in the plasma samples taken.

22. Raw data for the *in vivo* pharmacokinetic trial (goat model)

Diminazene (5 mg/kg):

Sample	Time (hrs)	Concentration (ng/ml)
2nd trough	95.97	900
3rd trough	143.97	840
30 mins	144.50	8637
1 hr	145.00	6017
2 hrs	146.00	5420
4 hrs	148.00	2893
8 hrs	152.00	2093
24 hrs	168.00	1258
48 hrs	192.00	965
96 hrs	240.00	861
192 hrs	336.00	568

DB 75 (2.5 mg/kg):

Sample	Time (hrs)	Concentration (ng/ml)
2nd trough	95.97	1750
3rd trough	143.97	1577
30 mins	144.50	2530
1 hr	145.00	2110
2 hrs	146.00	2227
4 hrs	148.00	2193
8 hrs	152.00	2120
24 hrs	168.00	1630
48 hrs	192.00	1706
96 hrs	240.00	2140
192 hrs	336.00	1600

DB 75 (1.25 mg/kg):

Sample	Time (hrs)	Concentration (ng/ml)
2nd trough	95.97	1915
3rd trough	143.97	2380
30 mins	144.50	2890
1 hr	145.00	3115
2 hrs	146.00	2740
4 hrs	148.00	2090
8 hrs	152.00	2475
24 hrs	168.00	1835
48 hrs	192.00	2070
96 hrs	240.00	2375
192 hrs	336.00	1540

DB 867 (2.5 mg/kg):

Sample	Time (hrs)	Concentration (ng/ml)
2nd trough	95.97	1610
3rd trough	143.97	1847
30 mins	144.50	3310
1 hr	145.00	3263
2 hrs	146.00	2813
4 hrs	148.00	2673
8 hrs	152.00	2390
24 hrs	168.00	2140
48 hrs	192.00	1910
96 hrs	240.00	1793
192 hrs	336.00	1683

DB 867 (1.25 mg/kg):

Sample	Time (hrs)	Concentration (ng/ml)
2nd trough	95.97	1877
3rd trough	143.97	2257
30 mins	144.50	3663
1 hr	145.00	3340
2 hrs	146.00	2933
4 hrs	148.00	2723
8 hrs	152.00	2533
24 hrs	168.00	2310
48 hrs	192.00	2067
96 hrs	240.00	1960
192 hrs	336.00	1637

DB 1192 (2.5 mg/kg):

Sample	Time (hrs)	Concentration (ng/ml)
2nd trough	95.97	28
3rd trough	143.97	46
30 mins	144.50	1563
1 hr	145.00	1020
2 hrs	146.00	474
4 hrs	148.00	278
8 hrs	152.00	203
24 hrs	168.00	78
48 hrs	192.00	40
96 hrs	240.00	23
192 hrs	336.00	10

DB 1192 (1.25 mg/kg):

Sample	Time (hrs)	Concentration (ng/ml)
2nd trough	95.97	17
3rd trough	143.97	19
30 mins	144.50	836
1 hr	145.00	738
2 hrs	146.00	170
4 hrs	148.00	108
8 hrs	152.00	69
24 hrs	168.00	23
48 hrs	192.00	18
96 hrs	240.00	18
192 hrs	336.00	9

Acknowledgements

Many individuals were involved in the success and completion of this dissertation of whom I am most grateful and appreciative to, in particular:

To my project supervisor, Professor Reto Brun for providing me with such an interesting and challenging topic, for trusting and allowing me the freedom to work and pursue my project at my own pace and ability. I thank him for letting me grow, change, make mistakes and for the many conferences, meetings, countries and people I have had the opportunity to attend, visit and meet in my time here. For his reassurance and advice during the harder moments and of course for putting me back on the right track whenever I strayed in the wrong direction. For his continued support and hard work, shown throughout my under- and post-graduate education, I am truly thankful and wish him only the best for his future.

To my external supervisor, Professor Philippe Büscher for always being there, for his outstanding support throughout my PhD and for the many wonderful laughs and conversations we have together! I thank him for his advice and wisdom and for the productive collaboration in the Canary Islands. I am perpetually impressed with him and his team in Antwerp and his aptitude to produce results of the highest calibre. With his fantastic sense of humour, there has never been a dull moment and I appreciate his belief in me and my capabilities.

To Professor Carlos Gutierrez for collaborating with me in the first place!! I thank him for offering his exceptional veterinarian skills and scientific understanding of our goat models and the immense success produced as a result of his hard work, continuous sample collection at all hours of the day and night and of course for his enthusiasm, motivation and wonderful sense of humour. I am most indebted to him, not only for broadening my scientific knowledge of the veterinary world, camels, goats and trypanosomes, but also for introducing me to the Canary Islands, its people, its customs and traditions and a new language! I am most grateful to him and his family for accepting me with open arms and making me feel so welcome and at home.

To Professor James Edwin Hall for his devotion to the diamidines, for his never-ending knowledge of each compound and its structure and to his superb team at the University of North Carolina. I would like to express my appreciation towards his warm and friendly attitude, which he has shown me since we first met. Through his endless ideas, precise explanations and deep understanding of pharmacokinetics, I have managed to collect a sneak preview into such a complex yet riveting aspect of drug discovery. It has been a pleasure working with him and I wish him all the best for the future.

To the staff and students of the Swiss Tropical Institute, especially to the Parasite Chemotherapy group for making me one of theirs and helping me with my project, to the administrative department for putting up with me and revealing the world of accounting to me, to Pascal Steiger and Karin Gysin for their *in vivo* assistance, care and interest in my project and to the fellow PhD students, who share our third floor office, for their jokes, hilarious conversations and fun moments both at work and in our free time ☺ Furthermore, I thank all the PhD and Master students, both at STI and outside, who have helped me in one way or another. I wish you all success with your projects and future careers.

A huge amount of gratitude must also be given to Stijn Deborggraeve (Bodo), Rosine Ali Ekangu, Titos Cau and Stijn Rogé for all the hard work, stressed time limits and excellent results produced for the PCR/*T. evansi* method used in the efficacy goat study. A special thanks to Bodo for allowing me to use his magic PCR/*T. evansi* method for my project!

In addition, I would like to express sincere gratitude to several individuals in America:

To Professor David Boykin for our many email conversations and for always finding time to answer my questions or check over some chemistry for me. It has been an honour to work with him and his drug synthesising team (who provided me with large amounts of DB 75, DB 867 and DB 1192 for the goat studies), enabling me to undertake the goat studies in the first place! Thank you.

To Professor Richard Tidwell for allowing me to test his compounds and for the pleasure of meeting him in Glasgow. It has also been an honour for me to work with him and his team, who no doubt put in much effort and hard work on my behalf and for that I thank them dearly, especially Susan Kilgore Jones for her never-fading enthusiasm and tolerance with the shipping of the goat samples, for the many compound structures and for my bombardment of emails to her, Huali Wu for an incredible amount of hard work, tediously sorting out the plasma samples and for performing the extractions and Arlene Bridges for her impressive scientific knowledge on mass spectrometry, her hard work and enthusiastic assistance with the analysis. Thank you for providing me with top quality PK data!

There are also some special people in the Canary Islands I would like to thank, who made my stay there so worthwhile:

To Maku for her amazing friendship, warm, open, caring personality and for letting me live with her, Kooka, Mixu and Taqi, during my time on the island. I thank her most for her deep trust and acceptance in me and for showing me the joys of Andalusian cooking, talking till the sun comes up and shopping! Jai guapa!!!

To Barbara, Natalie and family for making their home mine too. I adore them to pieces and I cherish our moments and experiences of Las Palmas nightlife, days out in the South and of course the work with the cows on the farm, dearly. Thank you for showing me another way of life, for including me in the good and bad times and for loving me the way you do. Te quierro mucho!

I dearly acknowledge the intense interest, constant help and facilities offered by the staff and students at the Hospital Clinicó Veterinario, without whom the goat work would not have been realised. I admire them greatly for the work they do and the wonderful parties and jokes they shared with me.

To all the staff and students of the Veterinary Faculty of the University of Las Palmas de Gran Canaria for their welcoming atmosphere and willingness to help me and involve me in their research.

Words can’t express the thanks I owe to my families and friends around the world, to those I have lost and to those I have found. I thank them for understanding and accepting the geographical distances between us. I treasure the moments where I can be with them and enjoy their company. To my close friends for all the things we do and share and for tolerating, supporting and loving me, even when you probably don’t want to! Thank you for letting me be me and for making me love you more and more each day, xxx!! I would like to extend my appreciation and admiration to my grandfather for keeping a watchful eye over my project and its developments, for our television moments together and for encouraging me to aim high. To both my grandmothers for their love and for leaving me with lasting memories of the good times I had growing up with them. To my wonderful parents for believing in me always, for their unconditional love and support and for providing me with only the best in life. To my gorgeous brother for his considerate and loving nature, his wit, charm and intelligence and for making me proud. In recognition of their dedication, hard work and constant motivation, including all the things they had to go through to get me this far, I dedicate this work to my parents and my brother. This is for you, love Kirsten xxx.

Curriculum vitae

Name	**Kirsten Gillingwater**
Nationality	**British / Swiss**
Date of birth	**8th January, 1980**
Place of birth	**Cambridge, UK**

Education, Research & Employment

2003 - 2007	**PhD in Cell Biology, Swiss Tropical Institute, Basel, Switzerland** (under the supervision of Professor Reto Brun)
Sept 2004	**LTK Module 1E: Introductory course in Animal Science, Institute for Laboratory Animal Studies, University of Zurich, Switzerland** (FELASA License, Category B)
June 2003	**Insecticide-treated bednet (pilot) study against visceral leishmaniasis on Marajó Island, Para, Brazil** (in accordance with Dr Clive Davies, London School of Hygiene and Tropical Medicine, London, UK and Dr Orin Courtenay, Warwick University, Coventry, UK)
2002 - 2003	**MSc in Medical Parasitology, London School of Hygiene and Tropical Medicine, London, United Kingdom** (taught course including a project in Amazonian region of north-eastern Brazil)
2000 - 2001	**Anti-leishmanial chemotherapy project on wild Spanish dogs, Swiss Tropical Institute, Basel, Switzerland** (in accordance with Dr Ronald Kaminsky, Animal Health Department, Novartis, Switzerland)
2000 - 2001	**Brunel Diploma for Professional Development and Education** (grade awarded: Distinction)
1998 - 2002	**BSc (Hons) in Medical Biology, Brunel University, Uxbridge, United Kingdom** (grade awarded: 2:1)
1991 - 1998	**St Mary's School, Cambridge, United Kingdom** (A-Level and GCSE at secondary school)

Conferences (participated & contributed)

2004

21st Annual Swiss Trypanosomatid Meeting, Leysin, Switzerland (29-31.01.04)

Trypanosomiasis & Leishmaniasis Seminar, České Budějovice, Czech Republic (27-30.08.04)

Annual Meeting of the Tropical Medicine & Parasitology (Swiss & German) Societies, Würzburg, Germany (23-25.09.04)

Swiss Society of Tropical Medicine & Parasitology, Münchwiler, Switzerland (14-15.10.04)

2005

22nd Annual Swiss Trypanosomatid Meeting, Leysin, Switzerland (27-29.01.05)

Non Tsetse Transmitted Animal Trypanosomoses meeting / NTTAT, Paris, France (21-22.05.05)

Non Tsetse Transmitted Animal Trypanosomoses meeting / NTTAT, Guangzhou, China (8-10.07.05)

12th International Congress on Protozoology / ICOP XII, Guangzhou, China (10-15.07.05)

2nd COST B22 Congress on Drug Discovery & Development for Parasitic Disease, Siena, Italy (29.09.05 - 01.10.05)

2006

23rd Annual Swiss Trypanosomatid Meeting, Leysin, Switzerland (2-3.02.06)

Non Tsetse Transmitted Animal Trypanosomoses meeting / NTTAT, Paris, France (20-21.05.06)

11th International Congress of Parasitology / ICOPA XI, Glasgow, Scotland (6-11.08.06)

2007

24th Annual Swiss Trypanosomatid Meeting, Leysin, Switzerland (8-10.02.07)

Non Tsetse Transmitted Animal Trypanosomoses meeting / NTTAT, Paris, France (19-20.05.07)

Lectures and courses given by the following professors were also attended during this post-graduate degree: Beck HP, Brun R, Burri C, Felger I, Keiser J, Pluschke G, Tanner M, Utzinger J and Weiss N.