DETERMINATION OF ACTIVITY OF MG-192 AGAINST INFECTIVE AFRICAN TRYPANOSOMES *IN VITRO* AND IN A MOUSE MODEL

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Determination of activity of MG-192 against infective African trypanosomes *in vitro* and in a mouse model

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

Signature…… Date…….

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DEDICATION

This thesis is dedicated to my family, especially my parents, Mr. and Mrs. Kamau and my brothers Albert and Michael.

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LIST OF ABBREVIATIONS

AAT	:	African animal trypanosomosis
AIDS	:	Acquired immunodeficiency syndrome
ANOVA	:	Analysis of variance
BBB	:	Blood-brain barrier
Bp	:	Base pairs
BRI	:	Biotechnology Research Institute
Bwt	:	Body weight
CAAT	:	Card agglutination test for trypanosomosis
CNS	:	Central nervous system
CSF	:	Cerebral spinal fluid
D.A	:	Diminazene aceturate
DALYs	:	Disability-adjusted life years
DBS	:	Dried blood spot
DMFO	:	DL-α-diflouromethylornithine
DNA	:	Deoxy-ribonuclease acid
dNTPs	:	Deoxynucleoside triphosphates
ED	:	Effective Dose
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme-linked Immunoassay
HAT	:	Human African Trypanosomosis
HCT	:	Haematocrit centrifugation technique
IACUC	:	Institutional Animal Care and Use Committee
IFN-γ	:	Interferon-gamma
Ig	:	Immunoglobulin
IMDM	:	Iscoves' modified dulbecco's medium
IP	:	Intraperitoneal
ISMM	:	Isometamidium chloride
ITS	:	Internal transcribed spacer
KALRO	:	Kenya Agricultural Livestock and Research Organization

KETRI	:	Kenya Trypanosomosis Research Institute
LAMP	:	Loop-mediated isothermal amplification
LDD	:	Last drug dose
LDL	:	Low-density lipoprotein
mAECT	:	mini-Anion exchange centrifugation technique
MIC	:	Minimum inhibitory concentration
M.W	:	Molecular weight
NECT	:	Nifurtimox-eflornithine combination therapy
NOAEL	:	No observed adverse effects level
ODC	:	Ornithine decarboxylase
PCR	:	Polymerase chain reaction
PCV	:	Packed cell volume
PSG	:	Phosphate saline- glucose
P.T.	:	Post-treatment
SAR	:	Structure-activity relationship
S.D	:	Standard deviation
SEM	:	Standard Error of the Mean.
SIT	:	Sterile insect technique
SRA	:	Serum-resistance associated
TCF	:	*Trypanosoma congolense* Forest
TCS	:	*Trypanosoma congolense* Savannah
TCK	:	*Trypanosoma congolense* Kilifi
TLF	:	Trypanosome lytic factor
TNF-α	:	Tumor necrosis factor- alpha
TAE	:	Tris-acetate ethylenediaminetetraacetic acid
VSG	:	Variant surface glycoprotein
WHO	:	World Health Organization

ABSTRACT

Treatment options for trypanosomosis are few and limited, complex to administer and have severe side effects. There is great need for discovery of new drugs that are safe, effective and easy to administer in rural settings. The structure-activity relationship theory (SAR) in drug discovery was explored in this study. This study investigated whether MG-192, a synthetic compound with similar structural features as Pentamidine, a standard trypanocidal drug, has the ability to clear African trypanosome infections. *In vitro* and *in vivo* activity of test compound MG-192 on *T. b. rhodesiense* and *T. congolense* isolates was investigated. Acute toxicity of MG-192 in mice was determined by injecting the mice with the test compound and observing them for 14 days. The *in vitro* cultures revealed that MG-192 has anti-trypanosomal properties, with an average MIC of 2.345×10^3 µg/ml ± 1.355 and 3.125×10^3 µg/ml for *T. b. rhodesiense* isolates KETRI 2537 and KETRI 2538. The *T. congolense* isolates KETRI 3867 and KETRI 3805 had an average MIC of 3.125×10^3 µg/ml and 3.516×10^3 µg/ml ± 0.552 respectively. Propagation of *T. b. gambiense* in donor mice did not yield sufficient parasitaemia and the experiment into the parasite could not proceed further. Studies into the acute toxicity effects of MG-192 in mice showed that after both single dose and multiple (5-day) intraperitoneal injections with various doses of MG-192, the no observed adverse effects level (NOAEL) was 1000 mg/kg. Single doses of MG-192 500 mg/kg and 200 mg/kg had an effect on mice body weight and PCV ($p < 0.05$). Period by drug interaction analysis on multiple administration of MG-192 1000 mg/kg also showed a significant effect on PCV ($p= 0.035$). The effect of 5-day administration of MG-192 on mice' organ weight: body weight ratio was significant in the kidneys (in the 200 mg/kg treatment group; $p=0.015$) and lungs (treatment groups 500 mg/kg; $p< 0.001$ and 1000 mg/kg; $p=0.038$). MG-192 did not clear trypanosome infections *in vivo* but single dose administration of the compound had a significant effect on parasitaemia ($p <0.001$) of *T. b. rhodesiense* KETRI 2537. The study revealed that the MG-192 had antitrypanosomal activity against trypanosomes *in vitro* but did not clear *T. b. rhodesiense* and *T. congolense* infections *in vivo* in concentrations of between 200 mg/kg and 750 mg/kg. The test compound, however, had a significant effect on parasitaemia patterns when mice infected with *T. b. rhodesiense* KETRI 2537 were

treated with a single dose of MG-192. More studies are needed to investigate the pharmacokinetic activity of MG-192 in mice in order to determine how to improve its efficaciousness in clearing trypanosome infections *in vivo*.

CHAPTER ONE

INTRODUCTION

1.1 Background

Trypanosomosis is a protozoan disease caused by extracellular parasites of the genus *Trypanosoma*. The disease is categorized into African trypanosomosis and American trypanosomosis and affects both humans and animals:

African trypanosomosis, also known as sleeping sickness, is transmitted by the bite of an infected tsetse fly and is present in many sub-Saharan countries (Shereni *et al.*, 2016). The causative parasite of African trypanosomosis is *Trypanosoma brucei*. The major subspecies of *T. brucei* that result in human disease, human African trypanosomosis (HAT) are *Trypanosoma brucei gambiense,* which causes chronic trypanosomosis and is found in West and Central Africa, while *Trypanosoma brucei rhodesiense,* which is found in East Africa, causes a more acute and more severe illness that can cause death in 6 months (Franco *et al*., 2014).

African animal trypanosomosis (AAT), also known as Nagana, is caused by *T. vivax*, *T. congolense* and *T. brucei brucei* and affect cattle, sheep, goats and wildlife (Muhanguzi *et al*., 2014). Other trypanosomes causing AAT include *T. evansi,* which infects camels and is transmitted by horse flies, *T. equinum*, which infects horses and *T.simiae,* a parasite that causes high fatality in domestic pigs (Nimpaye *et al*., 2011).

American trypanosomosis (Chagas disease) is present in Latin America and is caused by *Trypanosoma cruzi* parasite, which is transmitted to humans through the faeces of the triatomine bug (Chatelain, 2015).

Human African trypanosomosis (HAT) is a disease with two stages; haemolymphatic (early) stage and the meningoencephalitic (late) stage (Wang *et al*., 2008). Infection occurs when an infected tsetse fly bites an individual. Trypanosomes are released into the blood and lymph where they proliferate and cause symptoms such as intermittent fever, headache and lethargy. In the late stage of the disease, the trypanosomes cross the blood brain barrier, resulting in neurological abnormalities such as irregular sleeping patterns, loss of coordination and tremors. Progression of the disease sees the patients fall into a coma and death invariably results if chemotherapeutic intervention is not applied (Brun *et al.*, 2010).

Approximately 69.3 million persons are at risk of contracting HAT in sub-Saharan Africa (Simarro *et al.*, 2012). Livestock malnutrition, especially in pastoralist communities whose main source of livelihood is livestock, loss of agricultural productivity and overall economic losses make the search for an anti-trypanosome drug a priority (Giordani *et al.*, 2016).

Treatment for HAT is dependent on the causative subspecies and the disease stage in the afflicted individual (Barrett *et al.* 2007). For early-stage disease, Pentamidine and Suramin are the available drugs. Eflornithine (active against *T. b. gambiense* only) and melarsorprol (effective against both *T. b. gambiense* and *T. b. rhodesiense*) are the only available drugs for use in late-stage HAT disease (Kennedy, 2013). The use of drugs in combination, such as nifurtimox and eflornithine have also been considered in treatment of late-stage and melarsoprol-resistant trypanosomosis (Priotto *et al.* 2006). Diminazene aceturate, used to treat bovine trypanosomosis, is also used to treat humans in

trypanosome-endemic areas and has been found to be effective against early-stage *T. b. gambiense* and *T. b. rhodesiense* (Bacchi, 2009).

The development of resistance to trypanocidal drugs and the severe toxicity that occurs on administration of some of these drugs (Sands *et al.* 1985; Pepin *et al.* 1989; Anene *et al.* 2001; Barrett *et al.* 2007) make it imperative that new compounds are developed and researched, with the aim of producing a well-tolerated product that is effective against all forms of infective trypanosomes, at all stages of the disease.

1.2 MG-192

MG-192 is a synthetic compound, code-named so by its manufacturer (Dr. Joseph Chege). The compound was delivered to the laboratory in liquid form, with a maximum concentration of 10,000 mg/kg. It has an oily consistency and a molecular weight (M.W) of 192. MG-192 has a similar stereostructure to Pentamidine and has similar functional groups in its chemical composition (Dr. Joseph Chege, personal communication, October 8, 2012). The compound's chemical structure is yet to be patented and therefore its chemical structure cannot yet be revealed.

1.3 Pentamidine

Pentamidine is a diamidine compound and is administered as a treatment for early-stage *T. b. gambiense* disease, in addition to treating anti-fungal diseases (Barrett *et al.* 2007; Nolan *et al.* 1994). According to the structure-activity relationship (SAR) theory, compounds whose structure or functional groups are alike have similar biological activities/effects. One can therefore infer the pharmacological effects of a new compound from those of a known and existing drug with similar structures (McKinney *et al.* 2000). This creates a new avenue for drug discovery and development. It is

therefore suggested that MG-192 has similar pharmacological activity to Pentamidine and is a potential anti-trypanosomal drug.

1.4 Statement of the Problem

African trypanosomosis is a debilitating disease that has considerable negative effects on the socioeconomic development of affected communities. Treatment for the disease in both humans and animals is life-saving and is an effective control strategy against trypanosomosis. However, drug development for this neglected disease has not been a priority for pharmaceutical industries, yet the few options that exist are complex to administer in rural settings, have shown development of parasite resistance and result in severe side effects. These shortcomings warrant the need for more research geared towards development of compounds that are effective and well-tolerated in HAT patients. This study aims to discover the pharmacological activity of MG-192 against select trypanosomes that cause HAT and AAT.

1.5 Justification

There exists only five drugs for the treatment of HAT (Babokhov *et al.*, 2013) and three main compounds for control of AAT (Ilemobade, FAO). The lack of economic incentive for development of new drugs by pharmaceuticals coupled with development of drug resistance, severe toxicity and complex administration processes justify the need for development of new chemotherapeutic compounds against trypanosomosis. The test compound MG 192 could have anti-trypanosomal properties and with the demand for safe and effective therapeutics against pathogenic trypanosomes, it is imperative that experimental studies are conducted to evaluate its potential. The target market for MG-192, if proven effacacious, would be the at-risk human and animal populations. Furthermore, rational drug design of new lead compounds is an effective way of

determining the biological activity of a test compound against disease-causing organisms.

1.6 Research Questions

1. What is the efficacy of MG-192 as a trypanocidal drug against human and animal African trypanosomes?

2. What are the observable and measurable adverse side effects of MG-192 when administered in a mouse model?

3. Is MG-192 effective in eliminating trypanosomes in a laboratory model, in comparison to other reference drugs and does the compound have an effect, if any, on the parasitaemia patterns in infected mice treated with it?

1.7 Null Hypothesis

MG-192 has no anti-trypanosomal activity against *T. brucei rhodesiense*, *T. b. gambiense* and *T. congolense*.

1.8 Objectives of the study

1.8.1 General objective of the study

To investigate the efficacy and safety of MG-192 as a chemotherapeutic agent for use in the treatment of African trypanosomosis caused by *Trypanosoma brucei rhodesiense*, *T. b. gambiense* and *T. congolense*.

1.8.2 Specific objectives

1. To determine the Minimum Inhibitory Concentration (MIC) of MG-192 against *in vitro* cultures of *T. b. rhodesiense, T. b. gambiense* and *T. congolense*.
2. To identify the acute toxicity effects of MG-192 when administered in uninfected mice.
3. To determine the Effective Dose or ED 50 (The dose required to achieve 50% of the desired response in 50% of the test population) of MG-192 in mice infected with strains of *T. b. rhodesiense*, *T. b. gambiense* and *T. congolense* and the effect of MG-192 on parasitaemia patterns of the strains.

CHAPTER TWO

LITERATURE REVIEW

2.1 African Trypanosome Morphology

African trypanosomes are extracellular, uniflagellate protozoans that reside in the bloodstream of its hosts and undergo multiple changes in their form during their life cycle (Mathews, 2005).

Trypanosomes have a self-replicating mitochondrial DNA organelle, the kinetoplast (Pena-Diaz *et al.*, 2017). A single flagellum runs along the entire length of the parasite's body as a membrane and exits the cell body at the point where there is an invagination of the surface membrane, the flagellar pocket (Ooi and Bastin, 2013). The flagellar pocket is where nutrient intake, endocytosis and exocytosis occur. A series of microtubules under the cell membrane give the trypanosome its shape. The species *T. congolense* is unique has no free flagellum (Giordani *et al.* 2016).

African trypanosomes exhibit two main morphological forms; trypomastigote and epimastigote. The trypomastigote has a large kinetoplast located at the most posterior part of the body (postnuclear kinetoplast). The epimastigote form has the kinetoplast anterior to the nucleus, with a short cell membrane that runs along half the length of the body (Ooi and Bastin, 2013).

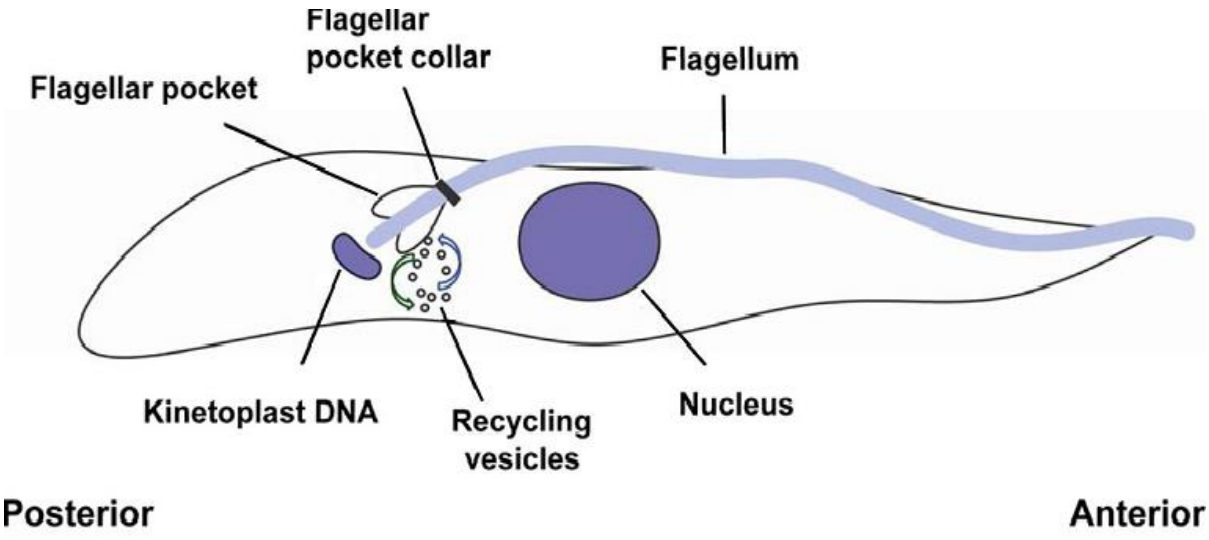

Figure 2.1: Morphological features of a Trypanosoma parasite, showing the salient organelles present in all trypanosomes (source; Rudenko, 2011).

2.2 Life Cycle of the African trypanosome

The lifecycle of a trypanosome occurs in two hosts; the tsetse fly and the animal or human host. (Fig 2.2). A tsetse fly becomes infected when it ingests the infected blood of a mammalian host. The bloodmeal contains short, stumpy forms of the parasite that differentiate into procyclic trypomastigotes in the midgut, as the parasites undergo cell division (Langousis and Hill, 2014). These forms then migrate to the proventiculus and into the salivary glands, where they undergo asymmetrical division into epimastigotes. Short epimastigote forms attach to the salivary glands' epithelium and replicate. They then differentiate into infective metacyclic trypomastigotes (Van Grinsven *et al.*, 2009).

When the infected tsetse fly feeds on a mammalian host, it injects the metacyclic trypomastigotes into the hosts' blood. The parasites multiply at the injection site and migrate through the lymphatic system and into the bloodstream of the host. They then transform into long, slender bloodstream forms (Langousis and Hill, 2014). These parasites migrate to various sites of the body, depending on their morphology and motility patterns (Bargul *et al.*, 2016). The long slender forms then differentiate into short, stumpy forms that are pre-adapted to the tsetse fly (Rico *et al.*, 2013).

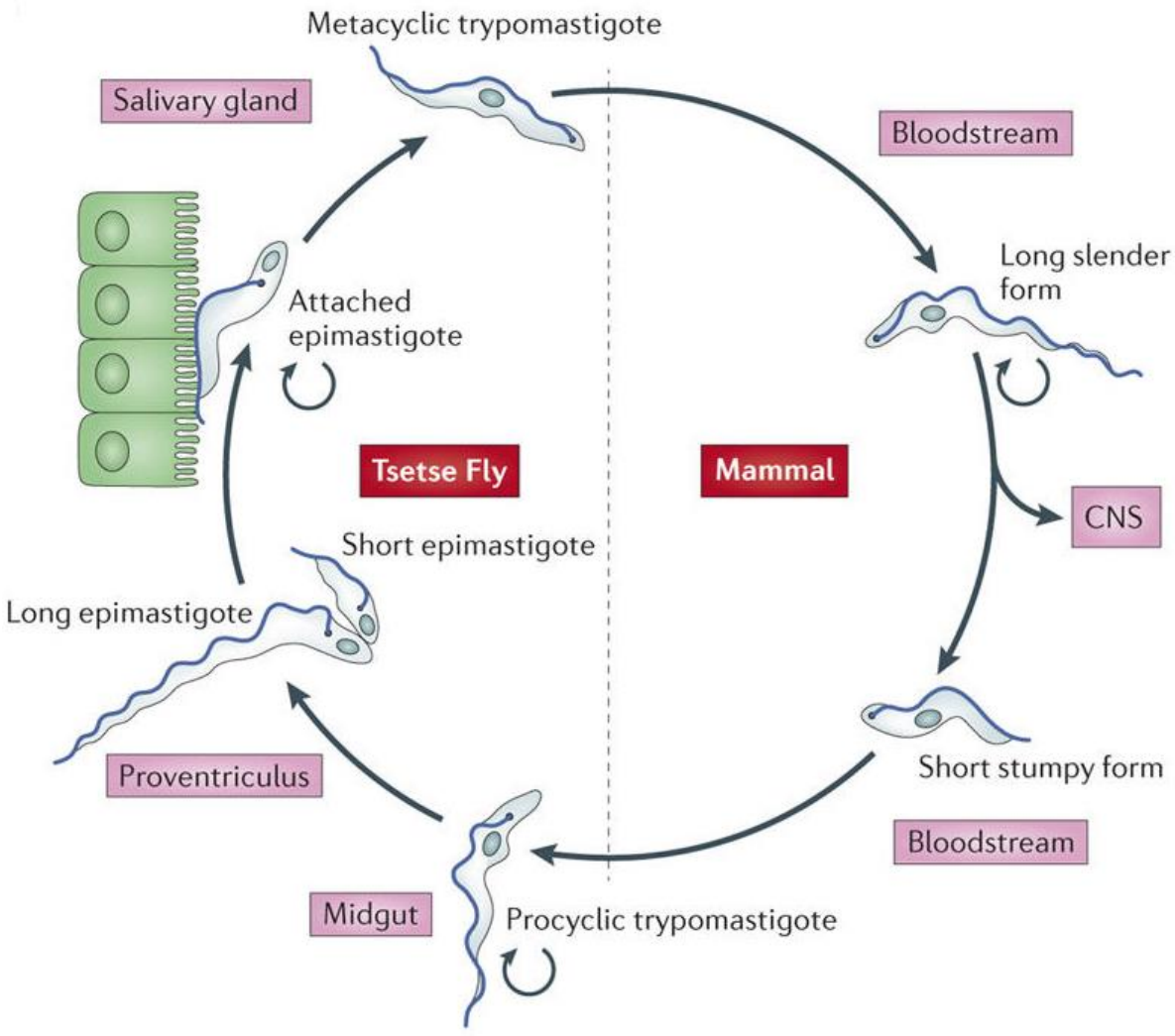

Figure 2.2: An image of the life cycle of a trypanosome parasite, showing the different morphological transformations of the parasite in the mammalian host and in the tsetse fly (source; Langousis and Hill, 2014**).**

2.3 Distribution and impact of African trypanosomosis

Human African trypanosomosis is restricted to sub-Saharan Africa, where its biological vector, the tsetse fly, is abundant. The main foci of *T. b .rhodesiense* transmission are southern Kenya, Malawi, Zambia, parts of Zimbabwe and southern Uganda, while high intensity transmission of gambiense HAT is present in Chad, Gabon, Congo, Democratic Republic of Congo, Angola, South Sudan, Central African Republic, Equatorial Guinea and north western Uganda (Simarro *et al*. 2013). The parasite *T. b. rhodesiense* is prevalent in cattle-herding and wildlife-protected areas, where the animals act as reservoirs of the parasite. At-risk populations include tourists, hunters, cattle herders and individuals living near the said areas. Limiting animal movement, early diagnosis and intervention of infected humans /animals and elimination of tsetse fly populations would curb transmission of HAT in these areas (Simarro *et al.* 2012).

An approximated 57 million people are at risk of acquiring gambiense HAT, while the at-risk population for rhodesiense HAT is 12.3 million (Simarro *et al.* 2012). The long duration of gambiense HAT infection in humans, despite the low vector competence of tsetse flies for *T. b.* gambiense, makes humans the main reservoir for *T. b. gambiense*. This human-tsetse fly-human transmission pattern is therefore the target of gambiense HAT control. This is carried out by early detection and treatment of all infected persons (Simarro *et al.*,2013).

Persons in non-endemic countries have been diagnosed with trypanosomosis. These are mostly tourists/travellers visiting endemic countries who acquire after visiting areas close to tsetse-infected wildlife. Approximately 84.4% of all HAT cases in these travellers are therefore because of the species *T. b. rhodesiense* (Urech *et al.*, 2011).

The number of HAT cases reported has steadily declined over the years. Approximately 8,000 infections were reported in 2012 (Wamwiri and Changasi, 2016). The impact of AAT, in East Africa alone, is estimated to cost US Dollars 4.5 billion (Shaw *et al.*, 2014). At least 46 million cattle and millions more sheep, goats, horses and camels are at risk of contracting the infection. The disease results in reduced production of meat and milk, decreased productivity, increased abortions, as well as lower crop yields (Kristjanson *et al*., 1999).

2.4 African animal trypanosomosis

The main causative agents of African animal trypanosomosis (AAT) are *Trypanosoma congolense*, *T. vivax*, *T. simiae*, and *T. suis* (Adams *et al*., 2010). This disease affects all domesticated animals, wild ruminants and most carnivores. *T. congolense* is the most important parasite in the aetiology of AAT and the parasite can be sub-typed into three groups; savannah, kilifi and forest (Isaac *et al*., 2016). The species *T. vivax*, *T. brucei brucei* and *T. congolense* infect most domesticated animals, while *T. suis* and *T. godfreyi* have a host preference in pigs. Parasite *T. simiae* predominantly affects pigs, but it has been detected in horses, camels and cattle. The species *T. evansi* is also pathogenic and causes disease in horses, camels and water buffaloes (Desquesnes *et al.*, 2013).

The pre-patent period of AAT, following infection, is between one to three weeks. A lesion (chancre) appears at the site of the tsetse bite. Here, the trypanosomes multiply and then enter the lymphatic system and finally enter the bloodstream. When parasitaemia begins, anaemia develops and at this first parasitaemia peak, fever is highest (Maudlin *et al*., 2004). At this peak, the lymph nodes and spleen become enlarged. Hypoglycaemia follows and the animal appears weak and lethargic. Death may occur a few weeks to months post-infection. However, most animals develop chronic AAT: The animal develops 'wasting syndrome' and becomes weak. Nasal discharge, lacrimation, difficult breathing, edema and change in the animal's hair colour are seen. Fertility problems in both male and female animals develop, such as abortion, with milk yields in cattle decreasing. Animals that recover clinically seem to relapse in many cases. At the terminal stage of the disease, cardiac problems are common and parasites may accumulate in the brain's capillaries, resulting in ischaemia. Chronic disease ends in death.

2.5 Human African trypanosomosis

Human African trypanosomosis is restricted to sub-Saharan Africa, where its biological vector, the tsetse fly, is abundant. The main foci of *T. b .rhodesiense* transmission are southern Kenya, Malawi, Zambia, parts of Zimbabwe and southern Uganda, while high intensity transmission of gambiense HAT is present in Chad, Gabon, Congo, Democratic Republic of Congo, Angola, South Sudan, Central African Republic, Equatorial Guinea and north western Uganda (Simarro *et al*. 2013).

The parasite *T. b. rhodesiense* is prevalent in cattle-herding and wildlife-protected areas, where the animals act as reservoirs of the parasite. At-risk populations include tourists, hunters, cattle herders and individuals living near the said areas. Limiting animal

movement, early diagnosis and intervention of infected humans /animals and elimination of tsetse fly populations would curb transmission of HAT in these areas (Simarro *et al.* 2012).

Gambiense HAT, a slow-progressing disease, relies on human-human transmission. Therefore, man is *T. b. gambiense*'s major reservoir. In order to prevent spread of the disease, early detection and treatment of all infected persons (healthy or otherwise) is paramount, in addition to applying vector control measures. Approximately 98% of all HAT cases are attributable to *T. b. gambiense* (Cnops *et al.*, 2016).

When an individual is bitten by an infected tsetse fly, a painful and itchy chancre develops at the site of the bite 5 to 15 days after inoculation (Kennedy, 2012). The swelling disappears after a few weeks. Around the same time, the patient starts developing intermittent fever and malaise. Recurrent erythematous patches develop as well as headaches, alongside oedema, anaemia, lymphadenopathy and weight loss. This is characteristic of the early (haemolymphatic) stage of the disease. Hypotension, heart murmurs and cardiomegaly may be present in individuals infected with *T. b. gambiense* (Blum *et al.* 2009).

When the parasites cross the blood-brain barrier (BBB), the disease enters the late stage and the parasite can be detected in neural tissue and cerebral spinal fluid (CSF). Signs of neurological injury become prominent at this stage. Tremors, loss of coordination and attention, somnolence and other sleep abnormalities, drooping of eyelids and general motor weakness are the major symptoms of this meningoencephalitic stage (Hide, 1999). Aggression, dullness, psychiatric disorders and inactivity become salient

and the patients develop dementia. The patient may slip into a coma at this stage and without treatment, death occurs.

2.6 Diagnosis of African Trypanosomosis

Before commencement of treatment for HAT, trypanosome disease must be staged and therefore CSF examination is imperative whenever CNS disease in suspected. Detection of trypanosomes in the early stage of the disease is relatively easy when compared to the late stage, which involves lumbar puncture. Presence of trypanosomes in CSF or increased protein content/white blood cell count is indicative of late stage disease (Bonnet *et al.,* 2015).

The gold standard for detection of trypanosome infection is by microscopic examination of peripheral blood, lymph node aspirates or cerebrospinal fluid (CSF) (Auty *et al.,* 2015; Bekele, 2015). Motile trypanosomes can be viewed by directly examining the fluid or by using stains, such as giemsa. Presence of *T. b. rhodesiense* is determined by observation of a thin or thick blood smear because parasitaemia levels remain high in the infected host (Kennedy, 2006). Direct microscopy is however unreliable because of its low sensitivity, especially in *T. b. gambiense*, due to its characteristic low, cyclic parasitaemia. Concentration methods, such as the haematocrit centrifugation technique (HCT), can however improve direct microscopy's reliability in trypanosome detection; the HCT method works by concentrating trypanosomes in the buffy coat of whole blood after centrifugation (Chappuis *et al.,* 2005). The mini-Anion exchange centrifugation technique (mAECT), which involves separation of trypanosomes from blood before visualization, significantly improves the sensitivity of light microscopy (Camara *et al.* 2010).

The card agglutination test for trypanosomosis (CATT) is a serological test used to screen for trypanosomal antibodies. It is a fast, affordable and sensitive test for use in endemic areas (Bonnet *et al.,* 2015). The CATT test works by agglutinating VSG-specific antibodies present in the test serum to the kit's antigens, which are isolated from the VSG coat of *T. b. gambiense* (Magnus *et al.* 1978). However, CATT has limited sensitivity (Kennedy, 2013) and other more sensitive serological tests are employed in HAT diagnosis. These include enzyme-linked immunoassays (ELISAs), complement fixation tests and immunofluorescence assays.

Molecular methods have been adapted for parasite detection in diagnosis of both HAT and AAT. Polymerase chain reaction (PCR) is both a sensitive and specific technique used to amplify trypanosome DNA. Primers specific to different trypanosome species have been developed and are used in PCR reactions to detect species-specific trypanosome infections (Masiga *et al.*, 1992; Wuyts *et al.*, 1994; Malele *et al.*, 2003; Njiru *et al.*, 2005).

The internal transcribed spacer (ITS1) PCR, which amplifies the ITS1 region of the ribosomal DNA (rDNA), is used as a universal test because it is highly sensitive to and detects all pathogenic trypanosomes. The ITS1-PCR's sensitivity is attributed to the highly conserved nature of the ITS1 region of the rDNA, as well as its small size (300-800 base pairs) (Thumbi *et al.*, 2008).

The serum resistance-associated (SRA) gene is the trait that confers pathogenic trypanosomes with resistance to lytic factors in human serum. The gene, present in *T. b. rhodesiense* only, has been used as a primer target and SRA-PCR is now used to detect *T. b. rhodesiense* infections (Gibson *et al.*, 2002).

The equipment needed to carry out conventional PCR and visualize PCR products in clinics hinders the use of the technique in rural areas/ field settings. The loop-mediated isothermal amplification (LAMP) method of DNA detection has been adopted as an alternative: This isothermal test requires only a water bath or heating block, used in place of a thermocycler. The test uses six to eight primers, targeting various DNA sites on the trypanosome genome. DNA amplification occurs by autocyclic strand DNA synthesis, using the enzyme *Bst* polymerase. Amplification product is visualized by various methods, such as observing for turbidity in the reaction tube or by using DNA intercalating dyes or probes (Njiru *et al.,* 2008). The LAMP method is highly sensitive and specific and a commercial kit has been developed for diagnostic purposes- the Loopamp *Trypanosoma brucei* kit (Eiken Chemical, Taito-Ku, Tokyo, Japan).

Other molecular methods, such as restriction fragment length polymorphism (RFLP) analysis for repetitive sequences of DNA have been developed for distinguishing *T. brucei* species (Hide *et al.*, 1994).

2.7 Treatment of African trypanosomosis

2.7.1 Treatment of human African trypanosomosis

Drugs available for treatment of trypanosome infections were developed more than 50 years ago (Bacchi, 2009). The only new addition is diflouromethylornithine (eflornithine), used to treat the meningoencephalitic stage of the disease. The resources needed to develop drugs against HAT, a disease endemic in poor populations, makes pharmaceutical companies shy away from their development. This impediment makes the illness a neglected disease. Two pharmaceutical companies, Bayer and Sanofi-Aventis, produce all the drugs licensed for HAT treatment and donate them to the World Health Organization (WHO) for distribution (Checchi and Barrett, 2008).

a) Pentamidine

Pentamidine is an aromatic diamidine used for treatment of early stage *T. b. gambiense* disease. The drug is administered by a series of seven to ten intramuscular injections (Barrett et al. 2007). Pentamidine has been used to treat *Pneumocystis carinii* pneumonia in patients with acquired immunodeficiency syndrome (AIDS). Pentamidine's mode of action in eliminating trypanosomes in humans is unknown, although it has been suggested that it works by promoting the cleavage of the parasite's minicircle DNA (Nagle *et al.*, 2014). The most common cytotoxic effect of Pentamidine administration is hypoglycaemia. Hypotension, pain at the injection site, leukopenia, changes in liver enzyme levels and thrombocytopenia are other adverse effects observed in patients (Sands *et al.* 1985).

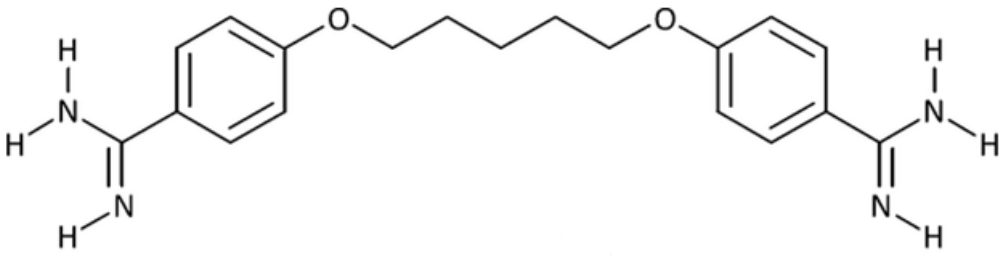

Fig 2.3 The Structural formula of Pentamidine

b) Suramin

Suramin is a polysulphonated naphthylamine compound used to treat early stage *T. b. rhodesiense* disease. The drug is administered by intravenous injection, with a total of five injections given over a period of four weeks (Barrett *et al.* 2007). Sumarin's mechanism of action has been linked to its ability to inhibit the activity of the parasite's

enzymes, such as thymidine kinase and glycolytic enzymes (Bacchi, 2009). The drug binds to plasma proteins in blood, including low-density lipoprotein (LDL), which is a major source of sterols for trypanosomes. The LDL is endocytosed by the trypanosome, directly delivering the drug to the parasite's system. Suramin does not penetrate the BBB, therefore it is not used in treatment of CNS disease. Toxicity is common and is characterized by neuropathy, renal insufficiency, neutropenia and bone marrow toxicity. However, these reactions are reversible (Brun *et al.* 2009).

c) Melarsoprol

Melarsoprol is a trivalent, organic, arsenic compound and is the most common drug in the therapy of late-stage HAT. It is effective against both Gambian and Rhodesian forms of the disease, although it is no longer a first-line treatment option for Gambian HAT. The drug is poorly soluble in water and has to be dissolved in propylene glycol. It is therefore administered as a single daily intravenous injection for 10 days or in two to four sets of three daily injections (Chappuis, 2007). The injections are extremely painful and lead to destruction of veins after repeated injections. The trypanocidal action of melarsoprol is not fully understood (Bacchi, 2009). The drug's adverse side effects are life threatening, with post-treatment reactive encephalopathy occurring in 10% of patients and in more than half of these cases, seizures, pulmonary edema and death result (Priotto, 2006). Pre-administration of oral prednisolone has been known to reduce the occurrence of melarsoprol-induced reactive encephalopathy in most patients. Convulsions, pyrexia, pruritis, arrhythmias and paraesthesia are other adverse side effects of the drug (Pepin *et al.* 1989). Treatment failure rates of 30% in some endemic areas after melarsoprol administration have been observed (Brun *et al.* 2009).

d) Eflornithine

Eflornithine (DL-α-diflouromethylornithine or DMFO), an inhibitor of enzyme ornithine decarboxylase (ODC), has been recommended for treatment of late-stage trypanosomosis caused by *T. b. gambiense* that is unresponsive to melarsoprol intervention (Hide, 1999). Administration of eflornithine is by four daily intravenous injections for fourteen days. This is due to its short plasma life (Bacchi, 2009). Eflornithine, which is taken up by passive diffusion, acts by degrading ODC enzyme in the parasite, thereby inhibiting polyamine biosynthesis and resulting in increased levels of S-adenosyl methionine, which causes unwanted methylation of cellular components such as proteins (Yarlett and Bacchi, 1988). This drug is not trypanocidal; it simply impedes multiplication of the trypanosomes. Side effects of this drug include bone marrow toxicity, leading to anaemia, thrombocytopenia and convulsions (Burri and Brun, 2003). However, it is still considered a safer, more effective drug to melarsoprol. Cost, availability and treatment duration limit the widespread use of eflornithine in trypanosomal intervention (Babokhov *et al.*, 2013). Drug combination therapies, involving eflornithine and other anti-trypanosomal drugs, have been tested and one in particular, nifurtimox-eflornithine combination therapy (NECT), has been approved as the standard first line of treatment for CNS-stage gambiense HAT therapy. This combination reduces both the dosage and toxicity of eflornithine in treated patients (Priotto *et al.*, 2009).

e) Nifurtimox

Nifurtimox, a nitrofuran derivative, is a drug used to treat American trypanosomosis (Chagas disease). It has been adopted for use in treating late-stage HAT treatment in cases where melarsoprol therapy has failed (Priotto *et al.* 2006). When used alone, the drug, which is administered orally, has average success (50%-80% cure rate) in treating

T. brucei gambiense disease (Pepin *et al*. 1989). Nifurtimox mechanism of action is its ability to generate a free radical and generate oxidative stress. Toxicity with nifurtimox use is characterized by nausea, vomiting, abdominal pains, among other adverse effects (Barrett *et al.* 2007).

f) Combination therapy

The use of more than one trypanocidal drug to simultaneously treat HAT has been brought on by treatment failure of monotherapeutics (Brun *et al.* 2001). The use of combination therapy has been known to have a synergistic effect that lowers the dosage of the drug(s), thus reducing toxicity and increasing efficacy. Development of resistance is also attenuated (Yun *et al*. 2010). Combinations of melarsoprol, eflornithine and nifurtimox have been clinically tested and have been found to increase efficacy of the drugs in comparison with their respective monotherapies (Brun *et al.* 2009). However, the use of melarsoprol in these combinations has been eliminated due to its adverse toxicity (Priotto *et al*. 2006).

The use of nifurtimox-eflornithine combination therapy (NECT) in treating second-stage *T. b. gambiense* has been demonstrated to have an excellent cure rate, with significantly fewer side effects and a shorter regimen when compared to administration of eflornithine alone (Yun *et al.* 2010).

g) Therapies in trial stages

Pafuramidine meleate (DB289) is a diamidine derivative given orally for the treatment of early stage HAT disease. However, development was halted after nephrotoxicity was observed during phase 3 of the drug's clinical trials (Barrett, 2010). Fexinidazole is another oral drug in development. It has shown efficacy in clearing *T. b. gambiense* in

late stage HAT disease in laboratory animals, with little toxicity observed (Torreele *et al.* 2010).

With advances in genome mapping and increased understanding of the trypanosome metabolic pathways, new molecular targets for drug development are being discovered. Characterization and validation of these targets will spur the development of new candidate drugs for the safe and effective treatment of HAT (Stuart *et al.* 2008).

2.7.2 Treatment of African animal trypanosomosis (AAT)

Diminazene aceturate (berenil) is used in treatment of bovine trypanosomosis and it has been used in unlicensed settings to treat human trypanosomosis. It has also been used in the treatment of babesiosis (Sivajothi *et al.,* 2016). Diminazene is an aromatic diamidine and its uptake into the trypanosome system is via the P2 nucleoside transporter. It is effective in treating early stage *T. b. rhodesiense* and *T. b. gambiense* infections (Bacchi, 2009). Its mechanism of action has been linked to its ability to interfere with RNA editing and trans-splicing, as well as its cleavage action on minicircle DNA (Kuriakose *et al.,* 2012). Little is published concerning toxicity of diminazene in humans, but it has been reported as being well-tolerated (Bacchi, 2009). Isometamidium chloride is a drug used for both prophylactic and therapeutic purposes against animal trypanosomiasis (Sahin *et al.,* 2014). Levels of isometamidium resistance in the field are higher than those of diminazene (Geerts *et al.,* 2001). Isometamidium is in the phenathridine class of compounds, along with homidium chloride, another treatment compound for *T. congolense* in cattle and *T. vivax* in pigs (Desquesnes *et al.,* 2013). Homidium chloride is mutagenic but it is still used in the field (Shiferaw *et al.,* 2015). Quinapyramine dimethylsulphate is a compound used in the treatment of *T. congolense* infections in camels and is administered subcutaneously (Gutierrez *et al.,* 2013).

2.8 Structure-activity relationship studies in drug development

Biological activity of a drug can be attributed to its structural features. One can therefore predict the pharmacological activity of a compound by comparing its structural/molecular features with a known drug with a similar structure (Sayes, 2014). This is the premise of the structure-activity relationship (SAR) theory. More complex SAR studies involving mathematical modelling have been used to design compounds with optimized biological activity and efficacy (Guha, 2013).

The current treatment for psychosis, ziprasidone, was developed from the chlorpromazine, a drug that calmed psychotic patients. Piroxicam, a major drug for the treatment of arthritis, was developed by SAR studies on 'oxicam' structures in compounds that were observed as being anti-inflammatory (Lombardino and Lowe, 2014). Evidence of the SAR theory in trypanosomosis can be seen in homidium chloride, used in AAT treatment, which is structurally related to isometamidium chloride and therefore have similar activity against *T. congolense* and even similar distribution properties in animals (Delespaux, 2010).

A number of Pentamidine analogues have been synthesized, with the hope of discovering a novel antitrypanosomal drug. Among them is furamidine, which is a prodrug of pafuramidine. Pafuramidine (DB-289) was found to have *in vitro* and *in vivo* efficacy against *T. b. rhodesiense* (Burri *et al.*, 2016).

2.9 Control of African trypanosomosis

Controlling the biological vectors of trypanosomes, that is, tsetse flies, is the ideal measure in curbing the spread of the parasite. The use of insecticides on domestic animals (cattle), as well as aerial and ground spraying of tsetse-infested habitats, may prevent transmission around homesteads (Torr *et al.* 2007). The control of tsetse fly

populations through the use of the sterile insect technique (SIT) has also been attempted: The SIT involves en masse release of sterilized male tsetse flies into the normal population over a long period of time. This gradually diminishes the reproductive capacity of the flies, ultimately reducing their overall population. The use of insect traps in combination with the sterile insect method helped eliminate trypanosomiasis from a Zanzibar island (Vreysen *et al.* 2000).

Actively seeking *T. b. gambiense*-infected persons (both clinical and sub-clinical cases) and administering prompt and effective treatment controls the West African HAT, since humans are the parasite's main reservoir. The screening of domestic animals in tsetse-endemic areas works in curbing transmission of the trypanosomes (Aksoy *et al.*, 2017). Disease surveillance techniques for use in field settings are wanting: Molecular techniques, such as conventional PCR, have high sensitivity and specificity but the requirement of expensive machinery impedes their use in rural settings. The use of isothermal LAMP technique is a good alternative to other DNA detection methods (Njiru *et al.* 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Area

The research study was done at the Kenya Agricultural Livestock & Research Organization's Biotechnology Research Institute (KALRO-BRI) in Muguga, Central Kenya.

3.2 Study Design

The research design used in this study was experimental. The study evaluated the ability of the compound MG-192 to clear various trypanosome infections and its acute toxicity effects in a mouse model. Four distinct experiments were carried out; *in vitro* drug efficacy test, *in vivo* acute toxicity tests, *in vivo* efficacy tests of the test compound against various infective trypanosome isolates and species identification of select isolates using polymerase chain reaction (PCR) assays.

3.3 Trypanosome isolates

Infective trypanosomes were selected from the KALRO-BRI biobank, as follows;

KETRI 2537, KETRI 2538 for *T. b. rhodesiense*: KETRI 2537 is an isolate derived from EATRO 1989, from patients in Uganda and serially passaged in monkeys (Fink and Schmidt, 1980). It is sensitive to diminazene aceturate and resistant to Suramin in mice systems (Bacchi *et al.*, 2004). KETRI 2538 is an isolate that was isolated in Mozambique and undergone 21 passages (Bacchi *et al.*, 1990) is multidrug-resistant (Murilla *et al.*, 2014).

KETRI 3805 and KETRI 3867 for *T. congolense*: KETRI 3805 is a clone of the isolate IL 3330 that was isolated from a cow and exhibits resistance to multiple drugs *in vitro*

and *in vivo* (Gray and Pergrine, 1993). KETRI 3867 is a field isolate that was isolated from a cow in the Njorore area of Coastal Kenya. The little-studied isolate is diminazene aceturate-sensitive *in vitro* (Unpublished data, Pharmacology Division of KALRO-BRI).

KETRI 3953, KETRI 3954, KETRI 3958, KETRI 3962, KETRI 3963 and KETRI 3966 for *T. b. gambiense*: These were all isolated from HAT-infected patients in South Sudan and were selected for this study because they were characterized (Maina *et al.*, 2007).

3.4 Laboratory Animals

Male Swiss white mice were used in this study, all approximately 6 weeks old and weighing between 20-35 grams (g). The mice were acquired from the KALRO-BRI small animal unit**.**

The mice were used as either donor mice - for trypanosome propagation- or experimental mice. Three donor mice were used for initial propagation of each trypanosome isolate. For the acute toxicity experiments, 6 mice per dose group and 4 mice per dose group were used for the single dose and multiple dose toxicity tests, respectively. Six mice per dose group were used for the efficacy tests and standardized protocols outlined in Eisler *et al.* (2001) were followed. Additional control groups of six mice were also included in each assay. A total of 239 mice were therefore used in this study.

3.5 Reference anti-trypanosomal drugs

The standard (reference) drugs used in this study were diminazene aceturate (Veriben®- Ceva, France) and Isometamidium chloride (Samorin®- Merial, France) for *T. congolense* assays; Pentamidine isethionate (Pentacarinat®- Mayer & Baker) for *T. b.*

gambiense tests; Suramin (Germanin®- Bayer, Germany) for *T. b. rhodesiense* tests and melarsoprol (Arsobal®- Aventis) for both *gambiense* and *rhodesiense* assays.

3.6 Ethical Approval

All protocols and guidelines pertaining to the use of mice in every experiment under this study were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Kenya Agricultural Livestock and Research Organization's Biotechnology Research Institute (KALRO –BRI) (Appendix 3).

3.7 Laboratory Methods

3.7.1 Maintenance of mice colony

On reception at the laboratory, the mice were treated with an intraperitoneal (IP) injection of 0.01 ml Ivermectin (Noromectin®- Norbrook) to get rid of any ecto- and endo-parasites and were then put in an experimental room and left to acclimatize for 14 days.

They were then put in cages with wood chippings as bedding material and fed on commercial mice pellets (Unga Limited, Kenya) and water *ad libitum*.

3.7.2 Preparation of test compound

The test compound, MG-192, was in liquid form and came from the manufacturer in the concentration of 10,000 mg/kg. The compound was diluted to a working solution of 5,000 mg/kg by adding 100 ml phosphate saline-glucose (PSG) solution to 100 ml MG-192 and gently shaking in order to homogenize it. Working solutions of 1000 mg/kg and 100 mg/kg were prepared in the same way and then stored in aliquots in dark bottles at room temperature and used within a week. Melarsoprol (Mel B) was re-constituted by diluting the drug using propylene glycol while Suramin, Diminazene aceturate,

Pentamidine and Isometamidium chloride were re-constituted by dissolving them in sterile distilled water.

3.7.3 Propagation and isolation of trypanosomes from cryopreserved isolates

Cryopreserved stabilates of *Trypanosoma brucei rhodesiense*, *T. b. gambiense* and *T. congolense* were retrieved from the KALRO-BRI biobank, where they had been stored in capillary tubes. The stabilates were left to thaw at room temperature, with each capillary tube immersed in 0.6 ml PSG solution. The stabilate-PSG solutions were then examined under a light microscope (magnification; ×40) to confirm viability of the revived trypanosomes, after which 0.2 ml of the trypanosome suspension was inoculated IP into a donor mouse. Each isolate was introduced into three donor mice for propagation. For the *T. b. gambiense* isolates, the donor mice were first immunosuppressed, using a modification of the method previously outlined by Maina *et al.* (2007). Briefly, a daily IP injection of cyclophosphamide was administered to the donor mice at a dose of 300 mg/kg body weight for four consecutive days prior to inoculation and a single injection every 10 days post-infection for 30 days. Parasitaemia was monitored daily by microscopic observation of a drop of tail blood, collected by disinfecting the tail with 70% ethanol, cutting its tip and putting a drop of blood on a clean, labelled microscope slide. The parasitaemia was estimated using the rapid matching method described by Herbert and Lumsden (1976).

A single donor mouse, positive for infection, was selected from each representative trypanosome species and strain (*T. b. rhodesiense* – KETRI 2537, *T. congolense* – KETRI 3867 and *T. b. gambiense* - KETRI 3953) and a drop of their tail blood preserved for molecular characterization, in accordance with the protocol outlined in Boid *et al.* (2009), as follows; each sample of tail blood was spotted on a labelled

Whatman filter paper (No.3) and the filter papers allowed to air dry for a few hours. The papers were then stored in sealable plastic bags with dessicant (silica gel) inside and stored in a cool, dry place at room temperature. The samples were preserved for species identification using PCR methods.

3.7.4 Identification of select trypanosome species by PCR assays.

Blood from representative isolates selected earlier in the study and preserved as described in Section 3.6.3 was re-constituted and DNA extracted from the dried blood spots. Polymerase chain reaction (PCR) tests were then done. The PCR test on *T. b. rhodesiense* isolate no. KETRI 2537 utilised primers complementary to serum resistance-associated (SRA) gene, a gene exclusive to *T. b. rhodesiense* species (Radwanska *et al.* 2002). The PCR on isolate KETRI 3867 used *T. congolense* primers (TCK/TCF/TCS) to identify its sub-group. Due to challenges in culturing and propagating the *T. b. gambiense* isolates, they were not included in subsequent experiments.

3.7.4.1 DNA extraction

A modification of the extraction method described by Boid *et al.* (1999) as well as the Qiagen DNeasy blood and tissue kit protocol was followed. Briefly, the dried blood spot (DBS) on the filter paper was cut using a sterile blade and placed in a labelled Eppendorf tube. Approximately 500 µl of PCR water was added to the Eppendorf tube containing the DBS and the tube incubated at 37˚C for 30 minutes (min). The tube was vortexed, after which the filter paper was removed. The eppendorf tube was then spun for 2 min at 12, 000 revolutions per minute (rpm) and the resulting supernatant was discarded. To the sediment in the tube, 200 µl AL buffer was pipette, followed by addition of 20 µl proteinase K. The mixture was thoroughly vortexed and then

incubated at 56˚C for 10 min, after which 200 µl absolute ethanol was added and vortexing was repeated. The mixture was pipetted into a DNeasy mini spin column and the column centrifuged at 8000 rpm for 1 min. The resulting flow-through was discarded along with the collection tube. With a new collection tube in place, 500 µl AW1 (wash) buffer was added to the spin column and centrifugation at 8000 rpm for 1 min followed. The flow-through and collection tube were discarded. After replacing the collection tube, 500 µl of AW2 buffer was pipetted into the spin column and the column centrifuged at 14,000 rpm for 3 min. The flow-through and collection tube were discarded and the spin column was placed in a sterile, labelled recovery (1.5 ml microcentrifuge) tube and 50 µl AE (elution) buffer pipetted into the center of the column. The column was the centrifuged at 8000 rpm for 1 min. The spin column was discarded and the elute in the recovery tube stored at 4˚C.

3.7.4.2 PCR amplification of trypanosome DNA

The polymerase chain reaction (PCR) tests done were the SRA PCR for the *T. b. rhodesiense* samples and *T. congolense* forest type, *T. c.* savannah type and *T. c.* kilifi type (TCF, TCS and TCK respectively) -specific PCR for the *T. congolense* isolate. The forward (F) and reverse (R) primers used for each PCR assay are indicated in Appendix 2.

An SRA PCR master mix with a final volume of 10 µl comprising 5X RT (reverse transcriptase) -buffer, 2mM magnesium chloride ($MgCl_2$) 200µM each of the four deoxynucleoside triphosphates (dNTPs), 1 µM each of the forward (SRA A) and reverse (SRA E) primers, 0.5 µl PCR water and 0.5 units of Taq DNA polymerase (Promega) was mixed with 4 µl template DNA and amplification was carried out in a GeneAmp® PCR system, Applied Biosystems, USA. PCR conditions were as follows: initial

denaturation at 95˚C for 3 min, followed by 35 cycles of 95˚C for 30 s, 60˚C for 30 s, 72˚C for 1 min and a final extension at 72˚C for 2 min.

The TCF, TCK and TCS PCR assays were conducted in a final reaction volume of 10 µl containing final concentrations of 2 µl 5× PCR buffer (= 1× PCR buffer), 200µ each of dNTPs, 2.5 mM $MgCl_2$, 0.5U Taq DNA polymerase, 2.9 µl PCR water, 1 µM of each forward and reverse primer for each species-specific PCR and 2 µl of the template DNA. The PCR conditions used in the amplification process for all the *T. congolense* PCRs were; initial denaturation at 94˚C for 1 min followed by 30 cycles of 92˚C for 30s, 60˚C for 45s, 72˚C for 45s and a final extension of 72˚C for 4 min.

3.7.4.3 Visualization of PCR products

The PCR products from the amplification assays were visualized by agarose gel electrophoresis. One gram of molecular grade agarose gel (Fischer Biotech) was dissolved in 50 ml Tris-acetate EDTA (TAE) buffer and heated. Ethidium bromide (2ml of 10 mg/ml) was added to the solution and after swirling gently to dissolve the ethidium evenly, the gel was left to set in a cast, with combs in it. The combs were gently removed from the poylmerized gel and 1× TAE buffer was added to a gel trough. Approximately 7 µl (100 bp plus, Fermentas) of a DNA ladder was loaded in the first well. The PCR products were then loaded and electrophoresis was done at 90 volts (V) for 40 min, after which the DNA bands (if any) were visualized in an ultraviolet (UV) trans illuminator. The size of the bands were then recorded.

3.7.5 Determination of *in vitro* activity of MG-192

Trypanosomes were harvested from the donor mice described in Section 3.7.3 and cultured in an enriched IMDM medium, containing various concentrations of the test compound and the reference drugs.

3.7.5.1 Preparation of culture medium

A modification of the method outlined in Baltz *et al.* (1985) was followed in preparation of the culture medium used in this experiment. Bloodstream trypanosome forms were cultured in Iscoves' modified dulbecco's medium (IMDM) supplemented with 25 mM Hepes medium, 3.024g/l sodium bicarbonate ($NaHCO_3$), soybean lipid, albumin and transferrin. The medium was further enriched with final concentrations of 0.2 mM 2-mecarptoethanol, 2 mM sodium pyruvate, 0.002mM bathocuproine sulfonate, 1.5 mM L-cysteine, 0.016 mM thymidine, 5υ penstrep solution and 20% (v/v) heat-inactivated fetal bovine serum (FBS).

3.7.5.2 Recovery of trypanosome parasites from donor mice

At first parasitaemia peak (antilog 8.1), the donor mice were euthanized by chloroform inhalation and blood drawn by cardiac puncture using a 23-gauge needle and a 1 ml syringe flushed with 10% ethylenediaminetetraacetic acid (EDTA), into a centrifuge tube.

3.7.5.3 Concentration of trypanosomes

The harvested blood (approximately 1ml) was diluted with 2 ml of the culture medium and the trypanosomes separated from the red blood cells by first performing centrifugation at 1000 rpm for 10 min and discarding the red pellet then repeating centrifugation on the filtrate, at 3000 rpm for 10 min and discarding the resulting supernatant. The pellet containing the parasites was re-suspended in 1 ml culture medium and gently shaken. The trypanosomes in solution were then counted using a haemocytometer and diluted to 1×10^5 trypanosomes per ml using the culture medium.

3.7.5.4 *In vitro* assay of anti-trypanosomal activity of MG-192

The assay to determine i*n vitro* activity of test compound MG-192 was performed in a 96-well flat-bottomed microtiter plate (Nunclon™Δ Surface-Nunc A/S, Denmark). Each test was carried out in duplicates in two adjacent rows of the plate, with drug exposure levels ranging from 0.001-100 µg/ml. Three tests were done in one microtiter plate and the border wells were not used. Firstly, 100 µl of culture medium was pipetted into wells of rows B-G and columns 2-10. Drug stock solution of 200 µl was added to column 11 wells (see Appendix 1). Serial dilution was then done by transferring 100 µl from wells of column 11 to wells of column 10 by use of a multichannel pipette. 100 µl was then transferred from wells of column 10 to those of column 9 and so forth. From the wells of column 4, 100 µl of the medium was discarded. Columns 2 and 3 served as control wells containing the complete medium only. To all the used (60) wells, 100 µl of trypanosome suspension was added, with a seeding density of 1×10^5 trypanosomes/ml. Diminazene aceturate, Isometamidium (for *T. congolense* isolates), Suramin, Pentamidine and Mel B assays (for *T. b. rhodesiense* isolates) were carried out concurrently, with each drug assay being assayed in a separate well plate. The plates with *T. b. rhodesiense* were incubated in 37˚C, 5% carbon dioxide (CO_2) humidified conditions for 72 hours (h) while *T. congolense* cultures were incubated at 34˚C in a humid, 5% CO_2 atmosphere for 72 h, the known optimum conditions for the parasites. At the end of incubation, the microtiter plates were observed under an inverted microscope at 100-fold. In every row, the highest dilution with no motile trypanosomes was determined. Concentration of the drug in that well was considered its minimum inhibitory concentration (MIC).

3.7.6 Evaluation of acute toxicity of MG-192 in mice

In order to determine the safe dose range of the test compound MG-192 and determine its No Observed Adverse Effects Level (NOAEL), single escalating doses ranging from 200 mg/kg to 5000 mg/kg body weight (bwt) were administered via IP route to grouped experimental mice. Control (untreated) mice acted as the baseline for the observations. The mice were monitored for changes in behaviour, body weight (bwt) and packed cell volume (PCV). The safety of multiple dose (5-day) regimens of MG-192 on mice was also evaluated by administering to mice groups via IP route doses ranging from 200 mg/kg to 1000 mg/kg bwt for 5 consecutive days. Pentamidine was also included in this experiment as a comparator (reference) drug and administered for 7 days, as per its use in clinical practice. Changes in bwt and PCV were recorded. Major internal organs of the mice used in the multiple dose experiment were harvested, weighed and changes in the organ weight to body weight ratio measured.

3.7.6.1 Single dose acute toxicity test

Thirty Swiss white mice, all male and six weeks old, were dewormed as described in Section 3.7.1 and randomly placed in five groups comprising 6 mice each; four of these were test groups and one control group. Each group was placed in a labelled individual cage. The mice were then labelled and after a 7-day acclimatization period, had their baseline (pre-treatment) body weight (bwt) and packed cell volume (PCV) recorded. Their body weights were measured using an analytical weighing machine while the following procedure was carried out to determine the PCV of each mouse; first, the tip of the mouse tail was disinfected with 70% ethanol, after which it was cut and blood from it collected into a heparinized capillary tube. The tube was then sealed with plasticin and centrifuged at 10,000 rpm for 5 minutes in a haematocrit centrifuge

(Naessens *et al.*, 2005). The PCV, which is the volume of red blood cells in an unclotted sample of blood, was read using a microhaematocrit reader .The day the PCV and bwt measurements were taken was considered as day 0 of the experiment.

The test groups were injected IP with 5000 mg/kg, 1000 mg/kg, 500 mg/kg and 200 mg/kg of MG-192 on day 0. The control group was injected with sterile phosphate-saline glucose (PSG). The mice were then observed for any signs of overt toxicity, such as lethargy, agitation, loss of gait, after 15 minutes, then after one hour, two hours, four hours and every 24 hours thereafter for 14 days. Their weights and PCV were also read twice a week for the duration of the observation. Mice that showed signs of distress were eliminated from the experiment.

3.7.6.2 Multiple dose acute toxicity tests

Forty male mice, all 6 weeks old, were put into 10 groups, each comprising 4 mice, and allowed to acclimatize for 7 days. The groups were as follows: Groups I and II; MG-192 at 1000 mg/kg, Groups III and IV; MG-192 at 500 mg/kg, Groups V and VI; MG-192 at 200 mg/kg, Groups VII and VIII (control groups); PSG, Groups IX and X; Pentamidine at 4 mg/kg (Table 3.1). On day 0, the baseline weights and PCV were recorded and treatment commenced then. The test groups were injected with the respective dosages of MG-192 for 5 consecutive days. The comparator drug, Pentamidine, was administered for 7 consecutive days, according to the standard practice of Pentamidine administration in the field. The control group was administered with PSG solution for 7 days. The mice were observed daily for any signs of distress or overt toxicity. Drug administration was halted if any signs of overt toxicity were observed during the test period. Bwt and PCV parameters of all the mice groups were measured and recorded 24h after the last injection, after which a single group of mice

from each drug dosage (Groups I, III, V, VII and IX) was euthanized. Each carcass was dissected and gross pathology carried out to determine the effect of the dose regimen on the mice' internal organs. The heart, liver, kidneys, spleen and brain of the mice were removed and weighed. The rest of the mice groups were observed for 14 days post-treatment, after which their bwt and PCV parameters were recorded again. The mice were then euthanized and gross pathology on internal organs carried out. The spleen, kidneys, liver, brain and heart were removed from the carcasses and weighed.

Table 3.1: Treatment regimen for MG-192 multiple dose acute toxicity test in mice.

Mice (Treatment) Groups	**Dose/Treatment administered**
Groups I & II	MG-192 1000 mg/kg x 5 days
Groups III & IV	MG-192 500 mg/kg x 5 days
Groups V & VI	MG-192 200 mg/kg x 5 days
Groups VII & VIII	Pentamidine 4 mg/kg x 7 days
Groups IX & X	Control; PSG solution (0.2 ml) x 7 days

Each experimental group had 4 mice. The compounds were administered by intraperitoneal injection and mice observed for 14 days. PSG – Phosphate saline glucose solution.

3.7.7 *In vivo* evaluation of the efficacy of MG-192 in clearing trypanosome infections

The ability of the test compound MG-192 to clear trypanosome infections in infected mice was tested. Infected mice were administered with single doses and multiple doses (a daily dose for 5 consecutive days) of MG-192 and observed for parasitaemia. The

mice were then screened for trypanosome infection for the next 60 days, after which any mouse without any trypanosome parasites detected in their blood was considered cured.

3.7.7.1 Assessment of *in vivo* single dose sensitivity of MG-192

A single strain of infective trypanosomes was selected for *Trypanosoma brucei rhodesiense* and *T. congolense*. The strains selected were KETRI 2537 and KETRI 3867, respectively. The strains were ordered from the cryopreservation biobank and revived, as described in Section 3.7.3. Two donor mice per strain were injected IP with 0.2 ml of the trypanosome solution. Tail blood from each mouse was drawn and observed daily under a microscope for parasitaemia. When parasitaemia was at its peak (antilog 8.1), blood from their tails was drawn and diluted using PSG solution to a level of 1×10^5 trypanosomes/ ml.

Experimental mice were acquired and for strain, 5 groups, comprising 6 mice per group, were selected and acclimatized for 7 days. The experimental groups were as follows: Treatment groups; Mel B; 10 mg/kg, Suramin; 40 mg/kg, MG-192; 750 mg/kg, 500 mg/kg, 200 mg/kg and control (untreated) group; PSG solution.

Each strain group was infected with 2.0 $\times10^4$ trypanosomes and 24 h later, treated according to their grouping. The mice were observed for parasitaemia daily for the first week, then twice weekly after that for 60 days. Parasitaemia determination was by microscopic examination of the tail blood of the experimental mice.

3.7.7.2 Assessment of *in vivo* multiple dose efficacy of MG-192

Experimental (treatment) groups and a control group of six mice each were selected and acclimatized in the experimental room for 7 days. Donor mice were infected with the preserved trypanosome strains, as described in the previous test. Experimental mice were then administered IP with 2×10^4 trypanosomes/ml of trypanosome solution from

the donor mice. Daily microscopic examination of the experimental mice' tail blood commenced until trypanosomes were seen. When all mice in the experimental groups had developed parasitaemia, treatment with MG-192 IP in the various mice groups as indicated in Tables 3.2 and 3.3 below began. The control groups (infected but untreated) were administered with 0.2 ml of PSG solution.

Parasitaemia levels in the mice were monitored daily for the first 7 days (after commencing treatment) and twice a week thereafter for the remainder of the 60-day observation period, by microscopic examination of tail blood. At this point, any mice that showed no parasitaemia was considered cured and the test compound at that particular dose deemed effective in clearing the trypanosome infection. The mice were subsequently euthanized via chloroform inhalation.

Table 3.2: Multiple dose treatment regimen for mice infected with *T. b. rhodesiense* KETRI 2537

Mice (Treatment) groups	**Regimen**
MG-192 , Group A	750 mg/kg × 5 days
MG-192, Group B	500 mg/kg × 5 days
MG-192, Group C	200 mg/kg × 5 days
Suramin (reference drug)	20 mg/kg × 5 days
Pentamidine (comparator drug)	4 mg/kg × 7 days
Control (untreated) group	Sterile PSG (0.2 ml) × 5 days

Mice were administered with respective doses of the test compounds by IP injection for 5 consecutive days. PSG- phosphate saline glucose

Table 3.3 Multiple dose treatment regimen for mice infected with *T. congolense* KETRI 3867

Mice (Treatment) groups	**Regimen**
MG-192 , Group A	750 mg/kg × 3 days
MG-192, Group B	500 mg/kg × 3 days
MG-192, Group C	200 mg/kg × 3 days
Diminazene aceturate (reference drug)	20 mg/kg × 1 day
Control (untreated) group	Sterile PSG (0.2 ml) × 3 days

Mice in each experimental group were administered with the respective concentration of the test compound for 3 days. PSG – phosphate saline glucose.

3.8 Data analysis

Data was entered into Excel worksheets (Microsoft, USA) and all results presented as Means ± Standard Deviation (std dev). Effect of test compounds on weight and PCV parameters, in comparison with the baseline values, was analysed using repeated measures analysis of variance (ANOVA). Response ratio of organ weight to body weight was first transformed (using negative log) to normalize the data and then analyzed using linear regression (GenStat 14th Edition – VSN Intl, UK). Interaction between period and drug was also analysed using GenStat's multiple linear regression model. Statistical significance was set at 5% level of significance ($\alpha = 0.05$).

CHAPTER FOUR

RESULTS

4.1 PCR Amplification of isolates

4.1.1 Amplification of *T. b. rhodesiense* isolate KETRI 2537

Visualization of the PCR product of the isolate KETRI 2537 by gel electrophoresis revealed an amplicon of between 400 and 500 base pairs (bp), after amplification using SRA-A and SRA-E primers (Fig 4.1).

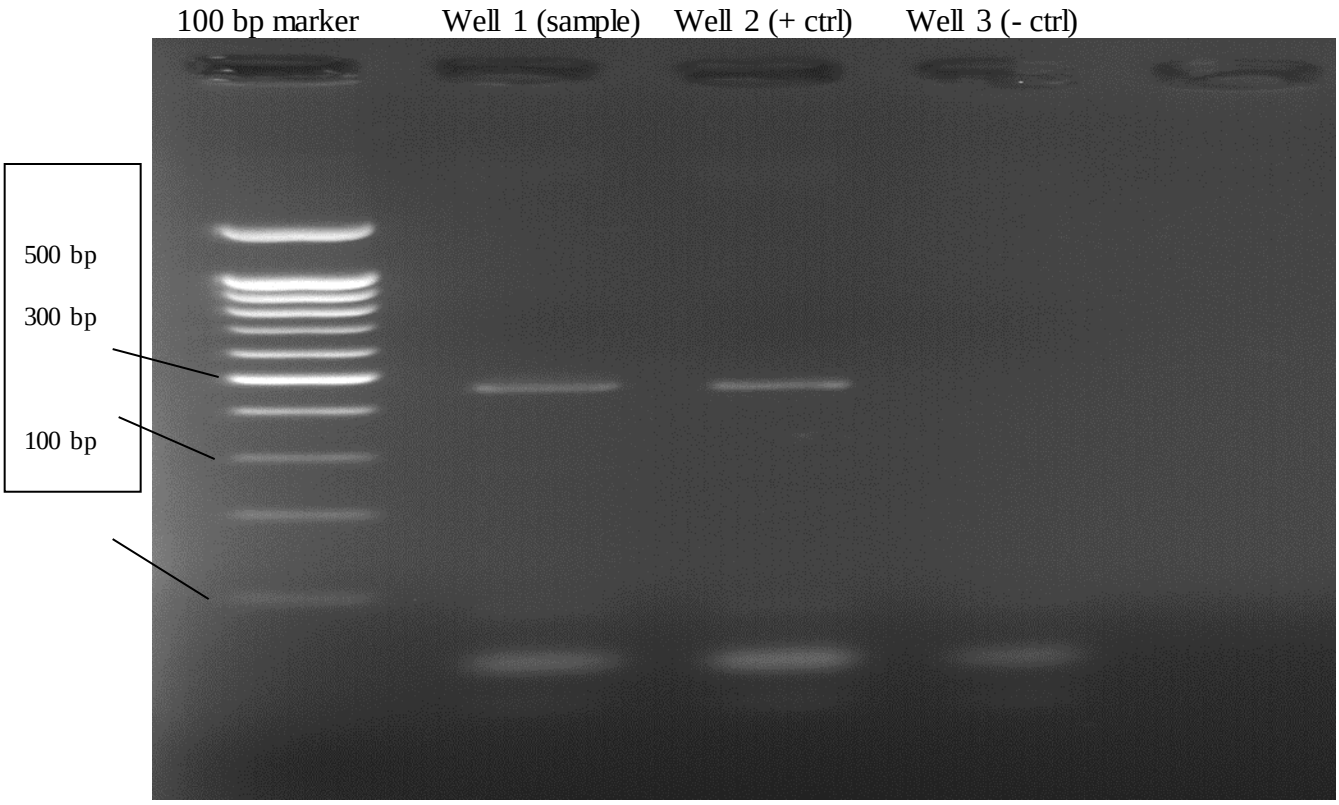

Fig 4.1: Gel electrophoresis of PCR product after amplification with SRA-A & SRA –E primers. Well 1 contained amplicon of *T. b. rhodesiense* KETRI 2537, which showed a band between 400 bp and 500 bp. Wells 2 and 3 contained the positive control and negative control respectively.

4.1.2 Amplification of *T. congolense* isolate KETRI 3867

Gel electrophoresis of isolate KETRI 3867 that was amplified using TCS 1 and TCS 2 primers revealed the amplicon to be approximately 300 bp in size (Fig 4.5).

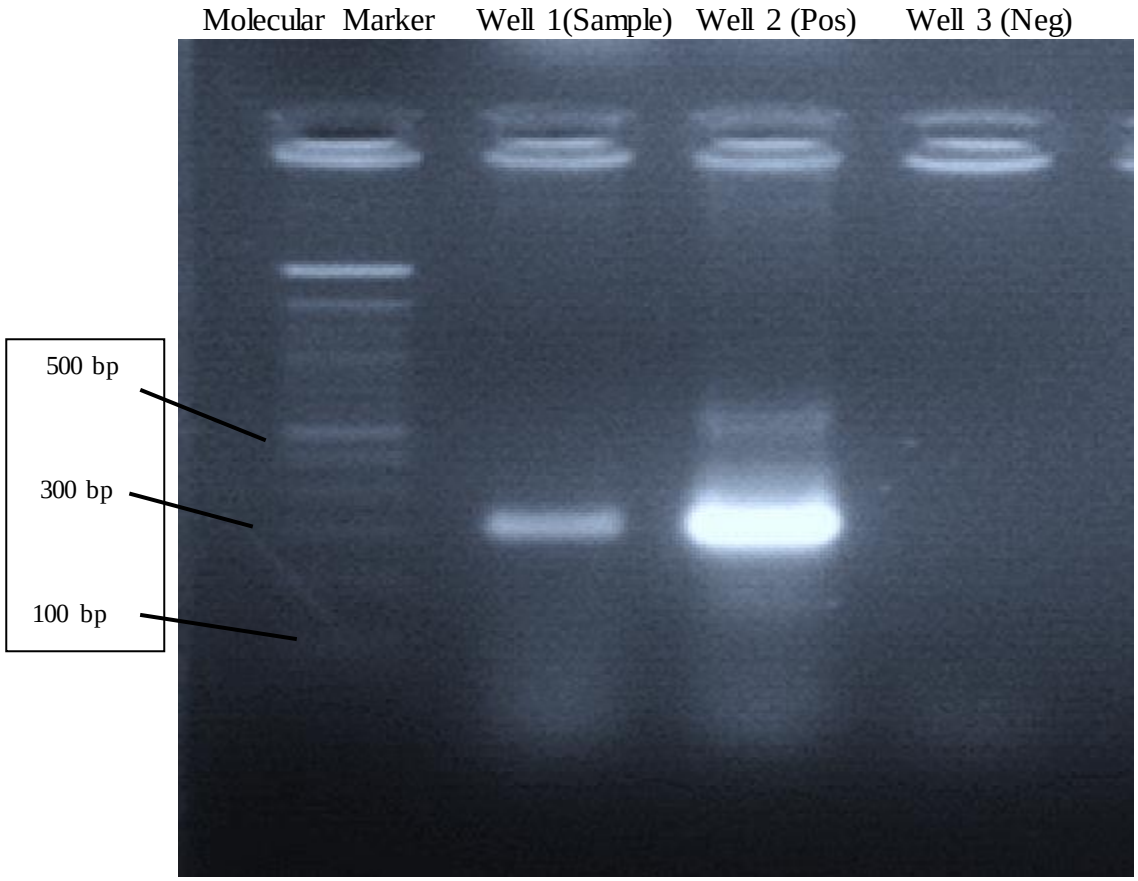

Fig 4.2: Gel electrophoresis image of PCR product after amplification with TCS1 and TCS2 primers. Well 1 contained amplicon *T. congolense* KETRI 3867, which had a band of approximately 300 bp. Wells 2 and 3 contained the positive (pos) control and negative (neg) control respectively.

4.2 *In vitro* sensitivity of MG-192 against *T. b. rhodesiense* and *T. congolense* isolates

The ability of the test compound MG-192 to inhibit proliferation of trypanosome isolates in culture media was tested and compared to those of standard trypanocides (Table 4.1).

The *in vitro* sensitivity tests were carried out in triplicates and results are shown in Table 4.1. The average minimum inhibitory concentration (MIC), the lowest concentration of a compound that prevents growth of a microorganism, was lowest for the standard (reference) drug against which the isolates were tested. For example, reference drug Mel B had an average MIC of 2.29×10^{-2} µg/ml when tested against the *T. b. rhodesiense* isolates, in comparison to test compound MG-192, which had an average MIC value of 2.345×10^{3} µg/ml. This indicates that these reference drugs are highly potent against the trypanosomes, even in low concentrations. The average MIC for the test compound MG-192 in all four isolates was between 2.345×10^{3} µg/ml and 3.516×10^{3} µg/ml.

Table 4.1: *In vitro* activity of MG-192 and standard trypanocides against *Trypanosoma brucei rhodesiense* and *T. congolense* isolates

Trypanosome isolate	Treatment compound	Average MIC of triplicate tests (± S.D)
T. b. rhodesiense KETRI 2537	Mel B	2.293×10^{-2} µg/ml (0.35)
	Suramin	13.54 µg/ml (6.505)
	MG-192	2.345×10^{3} µg/ml (1.355)
T. b. rhodesiense KETRI 2538	Mel B	2.293×10^{-2} µg/ml (0.358)
	Suramin	9.373 µg/ml (5.63)
	MG-192	3.125×10^{3} µg/ml
T. congolense KETRI 3805	Diminazene	57.3 µg/ml (9.007)
	ISMM	62. 5 µg/ml
	MG-192	3.516×10^{3} µg/ml (0.552)
T. congolense KETRI 3867	Diminazene	0.695 µg/ml
	ISMM	2.60 µg/ml (1.193)
	MG-192	3.125×10^{3} mg/ml

*MIC- Minimum Inhibitory Concentration, SD – Standard deviation, ISMM- Isometamidium chloride

4.3 *In vivo* acute toxicity studies

The acute toxicity profile of MG-192 in mice systems was determined by injecting mice intraperitoneally (IP) with various concentrations of the test compound, as a single dose and repeat (5-day) doses and observing the mice for signs of overt toxicity.

4.3.1 Single dose toxicity of MG-192 *in vivo*

4.3.1.1 Observable acute toxicity effects of MG-192 in mice

A control group of mice was administered with phosphate saline glucose (PSG) and used as a baseline reference during observation. Single dose administrations of MG-192; 750 mg/kg and 500 mg/kg had no observable signs of toxicity. However, one mouse that was administered with 1000 mg/kg of MG-192 developed instant paralysis, laboured breathing and wheezing. These symptoms, however, subsided half an hour after the injection. Administration of the highest concentration of MG-192 used in this study, 5000 mg/kg, caused instant death in 1 out of the 6 mice in that treatment group. Two other mice in the treatment group developed instant paralysis, laboured breathing and wheezing, which subsided eight hours after injection with the compound.

The No Observed Adverse Effects Level (NOAEL) dose for the *in vivo* single dose administration of MG-192 was 1000 mg/kg. The maximum dose of MG-192 used in this study, 5000 mg/kg, caused adverse effects on the respiratory and motor system of the experimental mice, resulting in temporary impairment and dyspnoea.

4.3.1.2 Effect of single dose administration of MG-192 on body weight

The lower concentrations of MG-192 used in this experiment resulted in a significant change in body weight ($p < 0.001$ and $p= 0.0026$ for treatment groups 200 mg/kg and 500 mg/kg respectively), when compared to the control group (Table 4.2). The mean change in weight after drug administration was positive in the mice in all test groups except the group treated with MG-192; 750 mg/kg. There was also no significant difference in the changes in weight in the highest concentration group, 750 mg/kg when compared with the control group, pre and post-administration of the drug. Interaction

between the treatment compounds and time was not significant in any of the treatment groups (p> 0.005), with reference to weight.

Table 4.2: Body weight changes in mice after single dose administration of MG-192

Treatment Groups of mice	Difference in means of body weight before and after treatment (± S.D)	p-value	Interaction of weight, time and treatment compound (p-value)
MG-192; 750 mg/kg	-0.75 (1.399)	0.287	0.056
MG-192; 500 mg/kg	1.38 (1679)	0.026	0.840
MG-192; 200 mg/kg	2.55 (1.565)	< 0.001	0.366
Control (PSG solution)	2.3 (0.627)	-	-

Mean differences of the control group were compared to the mean differences of the other treatment groups in order to determine their p-value. The p-value in the last column (period by drug interaction) represents the significant interaction of time, weight and test compound. SD – standard deviation, PSG – phosphate saline glucose.

4.3.1.3 Effect of single dose administration of MG-192 on PCV levels

The mean changes in PCV levels of mice in the experimental groups were found to be positive in the mice groups treated with the lowest concentration of MG-192, at 200 mg/kg (Table 4.3).

The statistical difference in the mean change in PCV pre- and post-treatment was only significant in the treatment groups of MG-192 200 mg/kg and 750 mg/kg (p=0.004 and 0.007 respectively), when compared with the control group.

Interaction of the drugs, PCV and time was not significant in any of the dose groups (p > 0.005).

Table 4.3: PCV changes in mice after single dose administration of MG-192

Treatment Groups of mice	Difference in means of PCV before and after treatment (± S.D)	p-value	Interaction of PCV, time and treatment compound (p-value)
MG-192; 750 mg/kg	-0.75 (3.685)	0.007	0.921
MG-192; 500 mg/kg	-1.33 (1.633)	0.446	0.742
MG-192; 200 mg/kg	2.60 (2.434)	0.004	0.051
Control (PSG solution)	0.25 (3.126)	-	-

Mean differences of the control group were compared to the mean differences of the other treatment groups in order to determine their p-value. The p-value in the last column (period by drug interaction) represents the significant interaction of time, PCV and test compound. PCV – packed cell volume. SD – standard deviation. PSG – phosphate saline glucose.

4.3.2 Multiple dose *in vivo* toxicity assays

4.3.2.1 Observation of mice after multiple dose administration with MG-192

No observable signs of toxicity were seen in any treatment group, whether immediately or 14 days after the last injection. Gross pathology was done to examine the major internal organs of the mice, and no signs of internal injury were seen, with the exception of one mouse under the Pentamidine treatment group at a drug concentration of 4 mg/kg, which had liver darkening and congestion.

4.3.2.2 Effect of multiple administration of MG-192 on body weight and PCV in experimental mice groups, 24 h and 14 days post-treatment

At 24 h post-last drug dose (LDD), there was a positive mean difference in the weight of all treatment groups, except for mice administered with 1000 mg/kg of MG-192, which showed a mean difference of -0.15 ± 0.801. Mice in the treatment group of Pentamidine, the comparator drug, showed a mean difference of 0.75 ±1.001, 24 h post-

LDD (Table 4.4). The weight differences for all treatment groups were however not statistically significant in comparison with the control group.

The PCV measurements taken 24 h post-LDD showed a negative mean difference in all treatment groups, with the highest difference seen in mice treated with 200 mg/kg of MG-192 (-3.5± 5.322). Pentamidine, the comparator drug, also resulted in a negative mean difference in mice administered with it (-2.75 ± 3.096). However, the PCV measurements 24-h post-LDD were not statistically significant when compared with the control group ($p>0.05$). Interaction of the test compounds, time and both weight and PCV parameters were not significant in any of the treatment groups (Table 4.4).

Table 4.4: Weight and PCV changes in mice, 24 h after multiple dose administration of treatment compounds

Treatment Groups of mice	Difference in means of weight, before and after treatment (± S.D)	p-value (Weight)	Interaction of weight, time and treatment compound (p-value)	Difference in means of PCV, before and after treatment (± S.D)	p-value (PCV)	Interaction of PCV, time and treatment compound (p-value)
MG-192 1000 mg/kg × 5 days	-0.15 (1.12)	0.927	0.948	-1.75 (6.02)	0.276	0.354
MG-192 500 mg/kg × 5 days	0.63 (0.43)	0.982	0.593	-1(1.732)	0.826	0.921
MG-192 200 mg/kg × 5 days	0.4 (1.122)	0.927	0.808	-3.5 (5.322)	0.276	0.698
Pentamidine 4 mg/kg × 7 days	0.75 (1.001)	0.963	0.722	-2.75 (3.096)	0.912	0.486
Control (PSG solution) × 7 days	-0.35 (0.854)	-	-	-0.5 (2.646)	-	-

Mean differences of the control group were compared to the mean differences of the other treatment groups in order to determine the p-values. PCV – packed cell volume. PSG – phosphate saline glucose solution. SD – standard deviation

The maximum acute effect of MG-192 on the weight and PCV of the experimental mice, 14 days after the last drug dose (LDD) was administered, is summarized in Table 4.5.

All treatment groups showed a positive mean difference in weight, 14 days post-LDD, with treatment group MG-192 500 mg/kg showing the highest mean difference (2.117 ±1.543).

None of the treatment groups of the experimental mice showed a significant difference in weight in comparison to the control (untreated) group ($p > 0.05$).

The mean differences in PCV of mice in treatment groups MG-192 1000 mg/kg and Pentamidine 4 mg/kg, 14 days post-LDD, were negative (-3.5 ±4.264 and -0.75 ±1.287 respectively) while those of MG-192 200 mg/kg and 500 mg/kg were positive (1.50 ±3.156 and 0.583 ±2.794 respectively). The difference in PCV pre- and post-treatment was statistically significant in mice of treatment group MG-192 200 mg/kg only, with a p-value of 0.015. Interaction of MG-192 at a concentration of 1000 mg/kg, PCV and time, was significant with days post-treatment ($p = 0.035$).

Table 4.5: Weight and PCV changes in mice, 14 days after multiple dose administration of treatment compounds

Treatment Groups of mice	Difference in means of weight, before and after treatment (± S.D)	p-value (Weight)	Interaction of weight, time and treatment compound (p-value)	Difference in means of PCV, before and after treatment (± S.D)	p-value (PCV)	Interaction of PCV, time and treatment compound (p-value)
MG-192 1000 mg/kg × 5 days	1.433 (0.956)	0.706	0.837	-3.5 (4.264)	0.186	0.035
MG-192 500 mg/kg × 5 days	2.117 (1.543)	0.826	0.597	0.583 (2.794)	0.386	0.848
MG-192 200 mg/kg × 5 days	2.02 (0.635)	0.894	0.849	1.50 (3.156)	0.015	0.356
Pentamidine 4 mg/kg × 7 days	1.675 (0.422)	0.827	0.835	-0.75 (1.87)	0.210	0.253
Control (PSG solution) × 7 days	1.216 (1.882)	-	-	0.25 (2.394)	-	-

Mean differences of the control group were compared to the mean differences of the other treatment groups in order to determine the p-values. PCV – packed cell volume, SD – standard deviation, PSG – phosphate saline glucose.

4.3.2.3 Organ weight to body weight ratio response following multiple dose administration of treatment compounds, 24 h and 14 days post-treatment

The effect of the drugs on the organ weight: body weight ratio, 24 h post-LDD, was significant in the kidneys (p= 0.015) in treatment group MG-192 200 mg/kg and in

lungs in treatment group MG-192 500 mg/kg ($p<0.001$) and group MG-192 1000 mg/kg ($p=0.038$), when compared with the control group (Table 4.6). Interaction between period and treatment compound was significant in the kidneys of mice in treatment groups of Pentamidine 4 mg/kg and MG-192 200 mg/kg ($p=0.046$ and $p=0.013$ respectively). Period by compound interaction was also significant in the liver of mice in treatment group Pentamidine 4 mg/kg ($p<0.001$) 14 days post-LDD.

Interaction between period and compound was significant in the lungs of mice treated with MG-192 500 mg/kg ($p=0.004$).

Table 4.6: Effect of multiple dose administration of MG-192 on organ weight: body weight ratio in mice groups; 24 hours and 14 days post-treatment

Organ	MG-192 1000 mg/kg	MG-192 500 mg/kg	MG-192 200 mg/kg	Pentamidine 4 mg/kg
Heart				
Effect of test compound (24h) (p-value)	0.330	0.552	0.489	0.446
Interaction of period and test compound (24 h vs. 14 days) (p'-value)	0.421	0.717	0.358	0.932
Liver				
Effect of test compound (24h) (p-value)	0.960	0.276	0.965	0.788
Interaction of period and test compound (24 h vs. 14 days) (p'-value)	0.754	0.381	0.952	<.001
Spleen				
Effect of test compound (24h) (p-value)	0.318	0.856	0.944	0.435
Interaction of period and drug (24 h vs. 14 days) (p'-value)	0.206	0.520	0.680	0.324
Kidneys				
Effect of test compound (24h) (p-value)	0.454	0.565	0.015	0.087
Interaction of period and test compound (24 h vs. 14 days) (p'-value)	0.427	0.328	0.013	0.046
Lungs				
Effect of drug (24h) (p-value)	0.038	<.001	0.353	0.282
Interaction of period and drug (24 h vs. 14 days) (p'-value)	0.207	0.004	0.236	0.462
Brain				
Effect of drug (24h) (p-value)	0.399	0.487	0.440	0.382
Interaction of period and drug (24 h vs. 14 days) (p'-value)	0.623	0.923	0.287	0.509

Mean differences of the control group were compared to the mean differences of the other treatment groups in order to determine their p-value. The p-value in the last

column (period by drug interaction) represents the significant interaction of time, weight and test compound. SD – standard deviation, PSG – phosphate saline glucose.

4.4 *In vivo* efficacy tests

The ability of the test compound, MG-192, to treat trypanosome infections in mice, was evaluated by injecting the different mice groups with different doses of the compound. The mice were then screened for the parasites for 60 days post- treatment.

4.4.1 Single dose efficacy of MG-192 in *T. b. rhodesiense* - infected mice

All mice in the treatment groups of MG-192 showed parasitaemia in their blood, 60 days post-treatment (PT). The cure rate of MG-192 at single doses between 200 – 750 mg/kg was therefore 0%. Mice treated with 40 mg/kg of Suramin (6 out of 6 mice) had no parasites in their blood by day 60 PT, therefore had 100% cure rate.

Survival rate of mice at the end of the 60-day PT period was 100% in treatment groups of MG-192 750 mg/kg, MG-192 500 mg/kg and Suramin 40 mg/kg. Two mice in treatment group MG-192 200 mg/kg died before the end of the observation period and therefore had a survival rate of 66.7%.

4.4.2 Single dose efficacy of MG-192 in *T. congolense*- infected mice

Mice in all treatment groups of MG-192 in this experiment showed parasitaemia, despite the treatments. Reference drug diminazene aceturate resulted in 100% cure rate, as all 6 mice in its treatment group showed no parasitaemia on microscopic observation of their tail blood. The mice in the treatment groups of MG-192 all died before the end of the observation period; the survival rate for mice treated with the compound MG-192 was therefore 0% at day 60, while survival rate of *T. congolense*- infected mice treated with Diminazene aceturate, the reference drug, was 100%.

4.4.3 Multiple dose efficacy of MG-192 in *T. b. rhodesiense* – infected mice

None of the mice in the treatment groups administered with five-day doses of MG-192 resulted were cured; all 6 mice in the groups maintained parasitaemia post-treatment. Mice treated with doses of Pentamidine at 4 mg/kg for 7 days also had a recurrence of parasitaemia by day 11 post-LDD and therefore had a 0% cure rate. Survival rate at day 60 post-LDD was 0% in the control (PSG) and in the treatment group of the highest concentration of MG-192, 750 mg/kg. Mice in treatment groups MG-192 200 mg/kg and 500 mg/kg had a survival rate of 16.7% and 66.7% respectively. Five out of six mice in the Pentamidine treatment group survived to day 60 post-LDD (83.3%), while the survival rate of the Suramin-treated group was 100%.

4.4.4 Multiple dose efficacy of MG-192 in *T. congolense*– infected mice

Treatment with various concentrations of MG-192 between 200 mg/kg and 750 mg/kg for 3 consecutive days did not clear the parasites from the experimental mice, therefore had a cure rate of 0%. Four out of six mice (66.7%) treated with diminazene aceturate at 20 mg/kg were cleared of parasitaemia, therefore the mice were considered to be cured.

4.5 Parasitaemia patterns following infection with trypanosomes and treatment with MG-192

4.5.1 Effect of single dose treatment with MG-192 on parasitaemia patterns of *T. b. rhodesiense* KETRI 2537

The pre-patent period of *T. b. rhodesiense* isolate KETRI 2537 innoculated in mice, then treated 24 hours later, decreased with increase in concentration of MG-192 (Table 4.7). The days it took for parasitaemia to develop to its peak in the mice was highest in the control group (31.8 days) and the lowest in MG-192; 750 mg/kg (18.7 days). The reference drug groups did not develop any parasitaemia in the 60 days post-treatment. A

significant effect on parasitaemia patterns of mice given the treatment compounds was seen when compared to the control group ($p < 0.001$).

Table 4.7: Parasitaemia patterns of *T. b. rhodesiense* KETRI 2537 following single dose administration with MG-192

	Pre-patent period (days)	**Days to initial parasitaemia peak (antilog ≥ 8.1)**	**p-value**
Drug/ Concentration Groups in Mice	**Median (range)**	**Mean (± S.D)**	
MG-192 750 mg/kg	6 (6)	18.7 (14.27)	< 0.001
MG-192 500 mg/kg	10.5 (6-24)	28 (9.92)	< 0.001
MG-192 200 mg/kg	17 (10-19)	22.6 (9.81)	< 0.001
Mel B 10 mg/kg	Nil	Nil	< 0.001
Suramin 40 mg/kg	Nil	Nil	< 0.001
Control (PSG solution)	6 (5-6)	31.8 (5.63)	-

Mice were infected with *T. b. rhodesiense* isolate KETRI 2537 and treated with various drug concentrations 24 h later. The pre-patent period and days to initial parasitaemia peak showed the effect of the compounds on parasitaemia patterns. S.D – standard deviation, PSG – phosphate saline glucose.

4.5.2 Effect of single dose treatment with MG-192 on parasitaemia patterns of *T. congolense* KETRI 3867

The median pre-patent period for the development of parasitaemia was the same in all the experimental mice (4 days). The days it took for the parasitaemia to peak after infection and treatment were fewest in the placebo group (4.8 days) and most in the group treated with MG-192; 200 mg/kg (Table 4.8).

The effect of the drugs on parasitaemia was significant when compared to the placebo (control) group only in the mice treated with diminazene ($p < 0.001$).

Table 4.8: Parasitaemia patterns of *T. congolense* KETRI 3867 following single dose administration with MG-192

	Pre-patent period (days)	**Days to initial parasitaemia peak (antilog ≥ 8.1)**	**p-value**
Drug/Concentration Groups in Mice	**Median (range)**	**Mean (± S.D)**	
MG-192 750 mg/kg	4 (1-4)	6.3 (2.33)	0.611
MG-192 500 mg/kg	4 (3-4)	5.3 (2.73)	0.122
MG-192 200 mg/kg	4 (1-4)	9.8 (5.42)	0.422
Diminazene 20 mg/kg	Nil	Nil	< 0.001
Placebo (PSG)	4 (3-4)	4.8 (0.408)	-

Mice were infected with *T. congolense* KETRI 3867 and treated with various drug concentrations 24 h later. The pre-patent period and days to initial parasitaemia peak showed the effect of the compounds on parasitaemia patterns. S.D – standard deviation, PSG – phosphate saline glucose.

4.5.3 Effect of multiple administration of MG-192 on parasitaemia patterns of *T. b. rhodesiense* KETRI 2537

The pre-patent period after administration of the treatment compounds was between 1 and 4 days for the mice administered with MG-192, while that of Pentamidine-treated mice was between 6 and 13 days after administration of the LDD.

The number of days to parasitaemia peak after administration of the compounds/ drugs was fewest in the placebo group (4.2 days) while the parasitaemia peak of antilog ≥8.1 was similar in the two highest doses of the test compound MG-192 (mean of 4.67 days). Suramin, the reference drug for Rhodesian trypanosomiasis treatment, cleared the parasites from the mice by the last day of treatment until the end of the study period.

The effect of the drugs on parasitaemia was significant when compared to the placebo (control) only in the mice treated with reference drugs Pentamidine and Suramin ($p < 0.001$).

Table 4.9: Parasitaemia patterns of *T. b. rhodesiense* KETRI 2537 following multiple (5-day) dose administration with MG-192

	2nd Pre-patent period (days)	**Days to initial parasitaemia peak post-treatment (antilog ≥ 8.1)**	**p-value**
Drug/Concentration Groups in Mice	**Median (range)**	**Mean (± S.D)**	
MG-192 750 mg/kg x 5 days	4 (1-4)	4.67 (1.03)	0.407
MG-192 500 mg/kg x 5 days	4 (1-4)	4.67 (1.03)	0.169
MG-192 200 mg/kg x 5 days	1 (1-4)	6.5 (5.21)	0.899
Pentamidine 4 mg/kg x 7 days	12 (6-13)	24.83 (4.02)	<0.001
Suramin 20 mg/kg x 5 days	Nil	Nil	<0.001
Placebo (PSG) x 5 days	1 (1)	4.2 (3.66)	-

Mice were infected with *T. b. rhodesiense* KETRI 2537 and after parasitaemia was established in all mice, treatment with the various concentrations of test compounds was done. The 2nd pre-patent period and days to initial parasitaemia peak showed the effect of the compounds on parasitaemia patterns. S.D – standard deviation, PSG – phosphate saline glucose.

4.5.4 Effect of multiple (3-day dose) administration of MG-192 on parasitaemia patterns of *T. congolense* KETRI 3867

The appearance of the *T. congolense* parasites in the mice after administration of the last dose of the treatment compounds was immediate, that is, on day 1(Table 4.10). The days to parasitaemia peak after administration of the last treatment dose were fewest in

MG-192; 200 mg/kg and MG-192; 500 mg/kg (mean of 3 and 3.8 days respectively). MG-192; 750 mg/kg delayed the number of days to development of parasitaemia to antilog ≥ 8.1 (mean of 11.2 days). Mice treated with diminazene aceturate; 20 mg/kg did not develop parasitaemia during the 60 days that parasitaemia was monitored for.

The effect of the drugs on parasitaemia of the infected mice was significant in the treatment groups diminazene ($p<0.001$) and MG-192; 500 mg/kg ($p<0.047$), when compared to the control/placebo group.

Table 4.10: Parasitaemia patterns of *T. congolense* KETRI 3867 following multiple (3-day) dose administration with MG-192.

	2nd Pre-patent period (days)	**Days to initial parasitaemia peak (antilog ≥ 8.1)**	**p-value**
Drug/Concentration Groups in Mice	**Median (range)**	**Mean (± S.D)**	
MG-192 750 mg/kg x 3 days	1(1-4)	11.2 (10.59)	0.389
MG-192 500 mg/kg x 3 days	1 (1-4)	3.8 (1.602)	0.047
MG-192 200 mg/kg x 3 days	1(1)	3 (1.55)	0.383
Diminazene 20 mg/kg x 1 day	Nil	Nil	<0.001
Placebo (PSG) x 3 days	1 (1-2)	9.6 (4.46)	-

Mice were infected with *T. congolense* KETRI 3867 and after parasitaemia was established in all mice, treatment with the various concentrations of test compounds was done. The 2nd pre-patent period and days to initial parasitaemia peak showed the effect of the compounds on parasitaemia patterns. S.D – standard deviation, PSG – phosphate saline glucose.

CHAPTER FIVE

DISCUSSION, CONCLUSION & RECOMMENDATIONS

5.1 Discussion

5.1.1 Determination of the minimum inhibitory concentration of MG-192

The *in vitro* cultures of infective trypanosomes incubated with MG-192 revealed that the compound has anti-trypanosomal properties that inhibited the proliferation of the *Trypanosoma brucei rhodesiense* and *T. congolense* isolates used in this study. However, the minimum inhibitory concentration (MIC) of MG-192 in these assays ($2.345\ x10^3$ µg/ml - $3.516\ 10^3$ µg/ml) was much higher than that of the reference drugs used in this study. This means that MG-192 is less potent than the existing trypanocidal drugs used as reference compounds in this experiment. The *in vitro* assay against *T. b. gambiense* was not carried out because the proliferation of the parasites in the donor mice did not achieve adequate levels of parasitaemia (≥ antilog 5.4) due to its fastidious nature (Brun *et al.*, 2001). Swiss white mice can first be immunosuppressed before infecting them with the parasite. During this study, adaptations of the method described by Maina *et al.* (2007) were used to immunosuppress and enhance the propagation of the *T. b. gambiense* isolate KETRI 3953 in Swiss white mice. After four repeated immunosuppression steps, parasitaemia levels remained below antilog 5.4. The experiment could therefore not proceed to the isolation and culture phase.

5.1.2 Acute toxicity effects of MG-192 in a mouse model

Intraperitoneal injection of mice with a single dose of the highest drug concentration of MG-192 in this study, 5000 mg/kg, resulted in instant death in one mouse and adverse toxicity symptoms (paralysis and laboured breathing) in four other mice. This implies

that MG-192, at high concentrations, has a negative effect on the respiratory and nervous system of mice systems. The no observed adverse effects level (NOAEL) after a single administration of the test compound was found to be 1000 mg/kg. However, one mouse in this test group developed paralysis and difficulty breathing, which subsided after half an hour. Perhaps the mouse was unable to effectively metabolise the test compound, resulting in the temporary, unexplained signs of overt toxicity. Examination of the experimental mice in the toxicity study revealed liver congestion in one mouse administered with Pentamidine. This correlates with studies that showed that Pentamidine alters liver functions in persons treated with it (Pepin and Khonde, 1996).

Single dose administration of concentrations of MG-192 had a significant effect on body weight of mice and PCV of mice administered with MG-192 200 mg/kg and 750 mg/kg ($p < 0.05$) when compared to the control group. There were no observable adverse effects of administering various concentrations of test compound MG-192 in mice for 5 consecutive days. The discrepancy in the weight observations between single dose and multiple dose administration of MG-192 suggests an increase in mice tolerance that reduced the effect of MG-192 after multiple dose administration and therefore with time, the effect of MG-192 on mice became insignificant.

At 14 days post-last drug dose, the PCV of mice in the lowest dose group of MG-192, 200 mg/kg, was significantly different than that of the control group ($p=0015$). Interaction of PCV, time and the concentration of MG-192 was also only significant in the highest concentration group of MG-192, 1000 mg/kg ($p=0.035$). These significant effects on PCV imply that MG-192 has some negative effect on the blood volume in mice, when administered either as a single dose or as repeat (multiple) doses for 5 days. There was a significant effect of repeat administration of MG-192 on the organ weight

to body weight ratio in mice administered with various concentrations of the compound: The ratio of kidneys to body weight of mice administered with 200 mg/kg of MG-192 showed a significant difference when compared to the control group. This suggests that at this low concentration, 24 h post-LDD, the mice system was yet to metabolise the test compound and therefore accumulation of MG-192 resulted in a negative effect on the kidneys. The kidneys of mice administered with pentamidine also showed a significant interaction of organ weight to body weight ratio, time and test compound when compared to the control group ($p=0.046$). This correlates with the knowledge that Pentamidine accumulates in the kidneys and that the drug may has adverse effects on kidneys (Poola *et al.*, 2003).

The effect of organ weight to body weight ratio of lungs in mice administered with MG-192 1000 mg/kg and 500 mg/kg was significant, 24 h after administration of the last dose. This implies that the test compound affects the respiratory system of mice. This can further be compared to the adverse effects of the high concentration of MG-192, 5000 mg/kg, which resulted in laboured breathing in the mice in its experimental group.

The interaction of liver weight to body weight ratio, time and dose of Pentamidine was significant in mice administered with it when compared to the control group. This correlates with the gross pathology observation of one of the mice in this test group that had a darkened liver. The two observations correlate with previous studies that show altered liver functions in persons treated with Pentamidine (O'brien *et al.*, 1997).

5.1.3 *In vivo* efficacy of MG-192 against trypanosomes

The test compound MG-192, at concentrations of between 200 mg/kg and 750 mg/kg, did not clear trypanosome infections in mice. In mice infected with *T. b. rhodesiense*

KETRI 2537, mice treated with a single dose of MG-192 750mg/kg and 500 mg/kg all survived until the end of the observation period. This could be an indicator that the test compound, at moderate to high concentrations, may increase survival rates in mice infected with *T. b. rhodesiense* parasites. None of the mice infected with *T. congolense* KETRI 3867 and treated with a single dose of MG-192 survived until the end of the observation period.

Treatment with concentrations of MG-192 500 mg/kg and 200 mg/kg for 5 consecutive days improved the survival rate of mice infected with *T. b. rhodesiense* KETRI 2537. The mice in the treatment group of MG-192 750 mg/kg all died. This can be attributed to the cumulative toxicity of the test compound, which may have led to decreased physiological activity within the mice and eventually, their deaths.

None of the mice infected with *T. congolense* KETRI 3867 and treated with concentrations of MG-192 for 3 days survived the entire 60-day observation period. Of the 6 mice treated with the reference drug diminazene at a concentration of 20 mg/kg, only four were cured of the trypanosome infection. This could mean that the *T. congolense* isolate KETRI 3867 has developed some resistance towards diminazene aceturate.

The administration of mice with a single dose of MG-192 after infection with *T. b. rhodesiense* KETRI 2537 had an effect on the days to the infection's first parasitaemia peak; however, MG-192 750 mg/kg had a lower mean of the days to parasitaemia peak than the other concentrations of the test compound; this may be due to physiological stress that the test compound may have exerted on the mice, resulting in weakened immunity of the mice. This may have given the parasites an opportunity to thrive.

Despite this, a single dose MG-192 had a significant effect on the overall parasitaemia patterns of parasite KETRI 2537, when compared to the untreated (control) group.

PCR analysis of *T. congolense* KETRI 3867 identified the parasite as belonging to the savannah sub-type. Prior to this study, the isolate had only been described by its morphology (Pharmacology Division, KALRO-BRI). The experiments carried out in this study revealed that KETRI 3867 is highly virulent, with parasitaemia levels peaking at 4 days in infected and untreated mice. Mice infected with this parasite strain all died by day 40 of the experiment, except for those treated with the reference drug, diminazene. Therefore, it can also be concluded that *T. congolense* KETRI 3867 is diminazene aceturate-sensitive.

Non-computational structure-activity relationship (SAR) theory has been successfully used in drug development and design. With the continued development of drug resistance and the constant search for safe and effective anti-trypanosomal drugs, the use of conventional methods of drug development and discovery is encouraged.

Key findings of this study of MG-192 revealed that MG-192 has *in vitro* potency against *T. b. rhodesiense* and *T. congolense* bloodstream forms *in vitro*. However, the compound, in its current form and composition, did not clear trypanosome infections in infected mice.

5.2 Conclusion

1. The test compound, MG-192, showed *in vitro* activity against cultures of *T. b. rhodesiense* KETRI 2537 and KETRI 2538 and *T. congolense* isolates KETRI 3867 and KETRI 3805, and had an average MIC average MIC of 2.345×10^3 µg/ml ± 1.355 and 3.125×10^3 µg/ml for *T. b. rhodesiense* isolates KETRI 2537 and KETRI

2538. The *T. congolense* isolates KETRI 3867 and KETRI 3805 had an average MIC of 3.125×10^3 µg/ml and 3.516×10^3 µg/ml ± 0.552 respectively.

2. The no observed adverse effects level (NOAEL) of MG-192 in mice, when administered as a single dose and as a repeat dose for 5 consecutive days was 1000 mg/kg. However, the test compound had a significant effect on body weight and packed cell volume (PCV) of some experimental mice. The kidneys and lungs of some mice were also affected by administration of MG-192 intraperitoneally.
3. MG-192 did not cure mice of *T. b. rhodesiense* KETRI 2537 infection or *T. congolense* KETRI 3867. The test compound, however, had a significant effect on parasitaemia patterns when administered as a single dose, in mice infected with *T. b. rhodesiense* 2537. Therefore, it had no effective dose level.

The null hypothesis was therefore accepted.

5.3 Recommendations

1. Further studies into how to effectively increase parasitaemia of gambiense infections in mice are needed, in order to study the parasite optimally.
2. Pharmacokinetic studies on the compound MG-192 need to be carried out in order to investigate how to improve its efficaciousness against trypanosome infections *in vivo*.

REFERENCES

Adams, E. R., Hamilton, P. B., & Gibson, W. C. (2010). African trypanosomes: celebrating diversity. *Trends in Parasitology*, *26*(7), 324–8. https://doi.org/10.1016/j.pt.2010.03.003.

Aksoy, S., Buscher, P., Lehane, M., Solano, P., & Van Den Abbeele, J. (2017). Human African trypanosomiasis control: Achievements and challenges. *PLOS Neglected Tropical Diseases*, *11*(4), e0005454. https://doi.org/10.1371/journal.pntd.0005454.

Anene, B. M., Onah, D. N., & Nawa, Y. (2001). Drug resistance in pathogenic African trypanosomes: what hopes for the future? *Veterinary Parasitology*, *96*(2), 83–100.

Auty, H., Torr, S. J., Michoel, T., Jayaraman, S., & Morrison, L. J. (2015). Cattle trypanosomosis: the diversity of trypanosomes and implications for disease epidemiology and control. *Revue Scientifique et Technique (International Office of Epizootics)*, *34*(2), 587–98.

Babokhov, P., Sanyaolu, A. O., Oyibo, W. A., Fagbenro-Beyioku, A. F., & Iriemenam, N. C. (2013). A current analysis of chemotherapy strategies for the treatment of human African trypanosomiasis. *Pathogens and Global Health*, *107*(5), 242–52. https://doi.org/10.1179/2047773213Y.0000000105.

Bacchi, C. J. (2009). Chemotherapy of human african trypanosomiasis. *Interdisciplinary Perspectives on Infectious Diseases*, 195040. https://doi.org/10.1155/2009/195040

Bacchi, C. J., Nathan, H. C., Livingston, T., Valladares, G., Saric, M., Sayer, P. D., Clarkson, A. B. (1990). Differential Susceptibility to DL-a-Difluoromethylornithine in Clinical Isolates of Trypanosoma brucei rhodesiense. *Antimicrobial Agents and Chemotherapy*, 1183–1188.

Baltz, T., Baltz, D., Giroud, C., Crockett, J., & Eisen, H. (1985). Cultivation in a semi-defined medium of animal infective forms of Trypanosoma brucei, T. equiperdum, T. evansi, T. rhodesiense and T. gambiense. *The EMBO Journal*, *4*(5), 1273–1277.

Bargul, J. L., Jung, J., McOdimba, F. A., Omogo, C. O., Adung'a, V. O., Krüger, T., Engstler, M. (2016). Species-Specific Adaptations of Trypanosome Morphology and Motility to the Mammalian Host. *PLOS Pathogens*, *12*(2), e1005448. https://doi.org/10.1371/journal.ppat.1005448.

Barrett, M. P., Boykin, D. W., Brun, R., & Tidwell, R. R. (2007). Human African trypanosomiasis: pharmacological re-engagement with a neglected disease. *British Journal of Pharmacology*, *152*(8), 1155–71. https://doi.org/10.1038/sj.bjp.0707354.

Barrett, M. P. (2010). Potential new drugs for human African trypanosomiasis: some progress at last. *Current Opinion in Infectious Diseases*, *23*(6), 603–608. https://doi.org/10.1097/QCO.0b013e32833f9fd0.

Bekele, E. E., & Begejo, B. (2015). The Current Situation and Diagnostic Approach of Nagana in Africa: A review. *Journal of Natural Sciences Research. 5*(17).

Blum, J. A., Schmid, C., Burri, C., Hatz, C., Olson, C., Fungula, B., Zellweger, M. J. (2009). Cardiac Alterations in Human African Trypanosomiasis (T.b. gambiense) with Respect to the Disease Stage and Antiparasitic Treatment. *PLoS Neglected Tropical Diseases*, *3*(2), e383.

Boid, R., Jones, T. W., & Munro, A. (1999). A simple procedure for the extraction of trypanosome DNA and host protein from dried blood meal residues of haematophagous Diptera. *Veterinary Parasitology*, *85*(4), 313–317.

Bonnet, J., Boudot, C., & Courtioux, B. (2015). Overview of the Diagnostic Methods Used in the Field for Human African Trypanosomiasis: What Could Change in the Next Years? *BioMed Research International*, *2015*, 1–10.

Brun, R., Blum, J., Chappuis, F., & Burri, C. (2010). Human African trypanosomiasis. *Lancet (London, England)*, *375*(9709), 148–59.

Brun, R., Don, R., Jacobs, R. T., Wang, M. Z., & Barrett, M. P. (2011). Development of novel drugs for human African trypanosomiasis. *Future Microbiology*, *6*(6), 677–691.

Brun, R., Schumacher, R., Schmid, C., Kunz, C., & Burri, C. (2001). The phenomenon of treatment failures in Human African Trypanosomiasis. *Tropical Medicine and International Health*, *6*(11), 906–914.

Burri, C., Yeramian, P. D., Allen, J. L., Merolle, A., Serge, K. K., Mpanya, A., Pohlig, G. (2016). Efficacy, Safety, and Dose of Pafuramidine, a New Oral Drug for Treatment of First Stage Sleeping Sickness, in a Phase 2a Clinical Study and Phase 2b Randomized Clinical Studies. *PLoS Neglected Tropical Diseases*, *10*(2), e0004362. https://doi.org/10.1371/journal.pntd.0004362

Camara, M., Camara, O., Ilboudo, H., Sakande, H., Kaboré, J., N'Dri, L., Bucheton, B. (2010). Sleeping sickness diagnosis: use of buffy coats improves the sensitivity of the mini anion exchange centrifugation test. *Tropical Medicine & International Health*, *15*(7), 796–799.

Chappuis, F., Loutan, L., Simarro, P., Lejon, V., & Büscher, P. (2005). Options for field diagnosis of human african trypanosomiasis. *Clinical Microbiology Reviews*, *18*(1), 133–46.

Chatelain, E. (2015). Chagas Disease Drug Discovery: Towards a New Era. *Journal of Biomolecular Screening*, *20*(1), 22–35. https://doi.org/10.1177/1087057114550585.

Cnops, J., Radwanska, M., & Magez, S. (2016). Immunopathology during African Trypanosomosis. *J Trop Dis*, *4*(4). https://doi.org/10.4172/2329-891X.1000197.

Delespaux, V., & Dekoning, H. (2007). Drugs and drug resistance in African trypanosomiasis. *Drug Resistance Updates*, *10*(1–2), 30–50.

Delespaux, V., Vitouley, H. S., Marcotty, T., Speybroeck, N., Berkvens, D., Roy, K., Van den Bossche, P. (2010). Chemosensitization of *Trypanosoma congolense*

Strains Resistant to Isometamidium Chloride by Tetracyclines and Enrofloxacin. *PLoS Neglected Tropical Diseases*, *4*(9), e828. https://doi.org/10.1371/journal.pntd.0000828.

Desquesnes, M., Dargantes, A., Lai, D.-H., Lun, Z.-R., Holzmuller, P., & Jittapalapong, S. (2013). Trypanosoma evansi and surra: a review and perspectives on transmission, epidemiology and control, impact, and zoonotic aspects. *BioMed Research International*, *2013*, 321237.

Desquesnes, M., Holzmuller, P., Lai, D.-H., Dargantes, A., Lun, Z.-R., & Jittaplapong, S. (2013). Trypanosoma evansi and surra: a review and perspectives on origin, history, distribution, taxonomy, morphology, hosts, and pathogenic effects. *BioMed Research International*, *2013*, 194176. https://doi.org/10.1155/2013/194176

Eisler, M. C., Brandt, J., Bauer, B., Clausen, P. H., Delespaux, V., Holmes, P. H., Geerts, S. (2001). Standardised tests in mice and cattle for the detection of drug resistance in tsetse-transmitted trypanosomes of African domestic cattle. *Veterinary Parasitology*, *97*(3), 171–82.

Fink, E., & Schmidt, H. (1980). Preclinical testing of potential trypanocidal drugs in primates. Preliminary investigation of an experimental diamidine in vervets. In J. Njogu, AR, Tukei PM, Roberts (Ed.), *Recent developments in medical research in eastern Africa. Proceedings of the 1st Annual Medical Scientific Conference of the Kenya Medical Research Institute and Kenya Trypanosomiasis Research Institute, 29 January to 1 February 1980, Nairobi, Kenya.* (pp. 173–182). Nairobi: Kenya Trypanosomiasis Research Institute.

Franco, J. R., Cecchi, G., Priotto, G., Paone, M., Diarra, A., Grout, L., Argaw, D. (2017). Monitoring the elimination of human African trypanosomiasis: Update to 2014. *PLOS Neglected Tropical Diseases*, *11*(5), e0005585.

Franco, J. R., Simarro, P. P., Diarra, A., & Jannin, J. G. (2014). Epidemiology of human African trypanosomiasis. *Clinical Epidemiology*, *6*, 257–75. https://doi.org/10.2147/CLEP.S39728.

Geerts, S., Holmes, P. H., Eisler, M. C., & Diall, O. (2001). African bovine trypanosomiasis: the problem of drug resistance. *TRENDS in Parasitology*, *17*(1).

Giordani, F., Morrison, L. J., Rowan, T. G., DE Koning, H. P., & Barrett, M. P. (2016). No Title. *Parasitology*, *143*(14), 1862–1889. https://doi.org/10.1017/S0031182016001268

Gray, M. A. A., & Peregrine, A. S. A. (1993). An in vitro assay for drug sensitivity of Trypanosoma congolense using in vitro-derived metacyclic trypanosomes. *Acta Tropica*, *54*(3–4), 291–300. https://doi.org/10.1016/0001-706X(93)90101-G

Guha, R. (2013). On exploring structure-activity relationships. *Methods in Molecular Biology (Clifton, N.J.)*, *993*, 81–94. https://doi.org/10.1007/978-1-62703-342-8_6.

Gutiérrez, C., González-Martín, M., Corbera, J. A., & Tejedor-Junco, M. T. (2013). *Chemotherapeutic agents against pathogenic animal trypanosomes*. (Méndez-Vilas A, Ed.). Formatex Research Centre.

Herbert, W. J., & Lumsden, W. H. (1976). Trypanosoma brucei: a 'rapid matching' method for estimating the host's parasitemia. *Experimental Parasitology*, *40*(3), 427–31.

Hide, G. (1999). History of sleeping sickness in East Africa. *Clinical Microbiology Reviews*, *12*(1), 112–25.

Ilemobade, A. Chemotherapy against African animal trypanosomiasis: Its strengths and limitations. Retrieved February 9, 2017, from http://www.fao.org/wairdocs/ilri/x5443e/x5443e0u.htm.

Isaac, C., Ciosi, M., Hamilton, A., Scullion, K. M., Dede, P., Igbinosa, I. B.,Turner, C. M. R. (2016). Molecular identification of different trypanosome species and

subspecies in tsetse flies of northern Nigeria. *Parasites & Vectors*, *9*(1), 301. https://doi.org/10.1186/s13071-016-1585-3.

Jacobs, R. T., Nare, B., & Phillips, M. A. (2011). State of the art in African trypanosome drug discovery. *Current Topics in Medicinal Chemistry*, *11*(10), 1255–74.

Kennedy, P. G. E. (2013). Clinical features, diagnosis, and treatment of human African trypanosomiasis (sleeping sickness). *The Lancet Neurology*, *12*, 186–194. https://doi.org/10.1016/S1474-4422(12)70296-X.

Kennedy, P. G. E. (2006). Diagnostic and neuropathogenesis issues in human African trypanosomiasis. *International Journal for Parasitology*, *36*(5), 505–512. https://doi.org/10.1016/j.ijpara.2006.01.012

Kristjanson, P. M., Swallow, B. M., Rowlands, G. J., Kruska, R. L., & De Leeuw, P. N. (n.d.). Measuring the costs of African animal trypanosomosis, the potential bene®ts of control and returns to research.

Kuriakose, S., Muleme, H. M., Onyilagha, C., Singh, R., Jia, P., & Uzonna, J. E. (2012). Diminazene Aceturate (Berenil) Modulates the Host Cellular and Inflammatory Responses to Trypanosoma congolense Infection. *PLoS ONE*, *7*(11), e48696. https://doi.org/10.1371/journal.pone.0048696

Langousis, G., & Hill, K. L. (2014). Motility and more: the flagellum of Trypanosoma brucei. *Nature Reviews Microbiology*, *12*(7), 505–518. https://doi.org/10.1038/nrmicro3274

Lombardino, J. G., & Lowe, J. A. (2004). A guide to drug discovery: The role of the medicinal chemist in drug discovery — then and now. *Nature Reviews Drug Discovery*, *3*(10), 853–862.

Magnus, E., Vervoort, T., & Van Meirvenne, N. (1978). A card-agglutination test with stained trypanosomes (C.A.T.T.) for the serological diagnosis of T. B. gambiense

trypanosomiasis. *Annales de La Societe Belge de Medecine Tropicale*, *58*(3), 169–76.

Maina, N., Maina, K. J., Mäser, P., & Brun, R. (2007). Genotypic and phenotypic characterization of Trypanosoma brucei gambiense isolates from Ibba, South Sudan, an area of high melarsoprol treatment failure rate. *Acta Tropica*, *104*(2–3), 84–90.

Malele, I., Craske, L., Knight, C., Ferris, V., Njiru, Z., Hamilton, P., Gibson, W. (2003). The use of specific and generic primers to identify trypanosome infections of wild tsetse flies in Tanzania by PCR. *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, *3*(4), 271–9.

Masiga, D. K., Smyth, A. J., Hayes, P., Bromidge, T. J., & Gibson, W. C. (1992). Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *International Journal for Parasitology*, *22*(7), 909–18.

Matthews, K. R. (2005). The developmental cell biology of Trypanosoma brucei. *Journal of Cell Science*, *118*(Pt 2), 283–90. https://doi.org/10.1242/jcs.01649.

McKinney, J. D., Richard, A., Waller, C., Newman, M. C., & Gerberick, F. (2000). The practice of structural activity relationships (SAR) in toxicology, *56*(1), 8–17.

Meyer, A., Holt, H. R., Selby, R., & Guitian, J. (2016). Past and Ongoing Tsetse and Animal Trypanosomiasis Control Operations in Five African Countries: A Systematic Review. *PLoS Neglected Tropical Diseases*, *10*(12), e0005247.

Muhanguzi, D., Picozzi, K., Hattendorf, J., Thrusfield, M., Kabasa, J. D., Waiswa, C., & Welburn, S. C. (2011). The burden and spatial distribution of bovine African trypanosomes in small holder crop- livestock production systems in Tororo District, south-eastern Uganda. https://doi.org/10.1186/s13071-014-0603-6

Murilla, G. A., Ndung 'u, K., Thuita, J. K., Gitonga, P. K., Kahiga, D. T., Auma, J. E., Ndung'u, J. M. (2014.). Kenya Trypanosomiasis Research Institute Cryobank for

Human and Animal Trypanosome Isolates to Support Research: Opportunities and Challenges. https://doi.org/10.1371/journal.pntd.0002747

Naessens, J., Kitani, H., Nakamura, Y., Yagi, Y., Sekikawa, K., & Iraqi, F. (2005). TNF-alpha mediates the development of anaemia in a murine Trypanosoma brucei rhodesiense infection, but not the anaemia associated with a murine Trypanosoma congolense infection. *Clinical and Experimental Immunology*, *139*(3), 405–10.

Nagle, A. S., Khare, S., Kumar, A. B., Supek, F., Buchynskyy, A., Mathison, C. J. N., Molteni, V. (2014). Recent Developments in Drug Discovery for Leishmaniasis and Human African Trypanosomiasis. *Chemical Reviews*, *114*(22), 11305–11347.

Nimpaye H, Njiokou F, Njine T, Njitchouang G R, Cuny G, Herder S, Cameroun. (2011). *Trypanosoma vivax, T. congolense* 'forest type' and *T. simiae*: prevalence in domestic animals of sleeping sickness foci of Cameroon. *Original Contribution Parasite*, *18*, 171–179.

Njiru, Z. K., Constantine, C. C., Guya, S., Crowther, J., Kiragu, J. M., Thompson, R. C. A., & Davila, A. M. R. (2005). The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. *Parasitology Research*, *95*(3), 186–192. https://doi.org/10.1007/s00436-004-1267-5

Njiru, Z. K., Mikosza, A. S. J., Armstrong, T., Enyaru, J. C., Ndung'u, J. M., & Thompson, A. R. C. (2008). Loop-mediated isothermal amplification (LAMP) method for rapid detection of Trypanosoma brucei rhodesiense. *PLoS Neglected Tropical Diseases*, *2*(1), e147. https://doi.org/10.1371/journal.pntd.0000147

Nolan, A., Lamey, P.-J., Macfarlanet, T. W., Aitchisons, T. C., Shaws, J., & Sirelj, J. Y. (1994). The effect of nebulised pentamidine on the concentration of intra-oral Candida albicans in H IV-infected patients. *J. Med. Microbiol*, *41*, 95–97.

O 'brien, J. G., Dong, B. J., Coleman, R. L., Gee, L., & Balano, K. B. (1997). A 5-Year Retrospective Review of Adverse Drug Reactions and Their Risk Factors in Human Immunodeficiency Virus-Infected Patients Who Were Receiving

Intravenous Pentamidine Therapy for Pneumocystis carinii Pneumonia. *Clinical Infectious Diseases*, *24*, 854–859.

Ooi, C.-P., & Bastin, P. (2013). More than meets the eye: understanding Trypanosoma brucei morphology in the tsetse. *Frontiers in Cellular and Infection Microbiology*, *3*, 71. https://doi.org/10.3389/fcimb.2013.00071.

Paliwal, S. K., Verma, A. N., & Paliwal, S. (2011). Neglected disease - african sleeping sickness: recent synthetic and modeling advances. *Scientia Pharmaceutica*, *79*(3), 389–428. https://doi.org/10.3797/scipharm.1012-08.

Peña-Diaz, P., Vancová, M., Resl, C., Field, M. C., & Lukeš, J. (2017). A leucine aminopeptidase is involved in kinetoplast DNA segregation in Trypanosoma brucei. *PLOS Pathogens*, *13*(4), e1006310. https://doi.org/10.1371/journal.ppat.1006310.

PEPIN, J., Guern, C., Ethier, L., Milord, F., Mpia, B., & Mansinsa, D. (1989). Trial of prednisolone for prevention of melarsoprol-induced encephalopathy in gambiense sleeping sickness. *The Lancet*, *333*(8649), 1246–1250. https://doi.org/10.1016/S0140-6736(89)92340-4.

Pépin, J., & Khonde, N. (n.d.). Relapses following treatment of early-stage *Trypanosoma brucei gambiens*e sleeping sickness with a combination of pentamidine and suramin. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *90*(2), 183–6.

Poola, N. R., Kalis, M., Plakogiannis, F. M., & Taft, D. R. (2003). Characterization of pentamidine excretion in the isolated perfused rat kidney. *Journal of Antimicrobial Chemotherapy*, *52*(3), 397–404. https://doi.org/10.1093/jac/dkg341

Priotto, G., Fogg, C., Balasegaram, M., Erphas, O., Louga, A., Checchi, F., Piola, P. (2006). Three Drug Combinations for Late-Stage Trypanosoma brucei gambiense Sleeping Sickness: A Randomized Clinical Trial in Uganda. *PLoS Clinical Trials*, *1*(8), e39. https://doi.org/10.1371/journal.pctr.0010039.

Priotto, G., Kasparian, S., Mutombo, W., Ngouama, D., Ghorashian, S., Arnold, U., Baudin, E. (2009). Nifurtimox-efl ornithine combination therapy for second-stage African Trypanosoma brucei gambiense trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. *The Lancet*, *374*, 56–64. https://doi.org/10.1016/S0140.

Radwanska, M., Chamekh, M., Vanhamme, L., Claes, F., Magez, S., Magnus, E., Pays, E. (2002.). The serum resistance-associated gene as a diagnostic tool for the detection of trypanosoma brucei rhodesiense. *American Journal of Tropical Medicine and Hygiene, 67(*6), 686-690.

Rudenko, G. (2011). African trypanosomes; the genome and adaptations for immune evasion. *Essays in Biochemistry*, *51*, 47–62.

Sahin, A., Asencio, C., Izotte, J., Pillay, D., Coustou, V., Karembe, H., & Baltz, T. (2014). The susceptibility of Trypanosoma congolense and Trypanosoma brucei to isometamidium chloride and its synthetic impurities. *Veterinary Parasitology*, *203*(3–4), 270–275.

Sands, M., Kron, M. A., & Brown, R. B. (1985). Pentamidine: a review. *Reviews of Infectious Diseases*, *7*(5), 625–634.

Sayes, C. M. (2014). The Relationships among Structure, Activity, and Toxicity of Engineered Nanoparticles. (2014). *KONA Powder and Particle Journal*, (31), 10–21. https://doi.org/10.14356/kona.2014002

Seeger, T., Seymour, P., Schmidt, A., Zorn, S., Schulz, D., Lebel, L. & Lowe, J. (1995). Ziprasidone (CP-88,059): a new antipsychotic with combined dopamine and serotonin receptor antagonist activity. *Journal of Pharmacology and Experimental Therapeutics.*, *275*(1).

Shaw, A. P. M., Cecchi, G., Wint, G. R. W., Mattioli, R. C., & Robinson, T. P. (2014). Mapping the economic benefits to livestock keepers from intervening against bovine trypanosomosis in Eastern Africa. *Preventive Veterinary Medicine*, *113*(113), 197–210. https://doi.org/10.1016/j.prevetmed.2013.10.024

Shereni, W., Anderson, N. E., Nyakupinda, L., & Cecchi, G. (2016). Spatial distribution and trypanosome infection of tsetse flies in the sleeping sickness focus of Zimbabwe in Hurungwe District. *Parasites & Vectors*, *9*(1), 605. https://doi.org/10.1186/s13071-016-1879-5.

Shiferaw, S., Muktar, Y., & Belina, D. (2015). Journal of Parasitology and Vector Biology A review on trypanocidal drug resistance in Ethiopia, *7*(4), 58–66. https://doi.org/10.5897/JPVB2015.0190.

Simarro, P. P., Cecchi, G., Franco, J. R., Paone, M., Diarra, A., Ruiz-Postigo, J. A., Jannin, J. G. (2012). Estimating and Mapping the Population at Risk of Sleeping Sickness. *PLoS Neglected Tropical Diseases*, *6*(10), e1859.

Simarro, P. P., Franco, J. R., Cecchi, G., Paone, M., Diarra, A., Ruiz Postigo, J. A., & Jannin, J. G. (2012). Human African Trypanosomiasis in Non- Endemic Countries (2000–2010). *Journal of Travel Medicine*, *19*(1), 44–53. https://doi.org/10.1111/j.1708-8305.2011.00576.x

Simarro, P., Franco, J., Diarra, A., Ruiz Postigo, J., & Jannin, J. (2013). Diversity of human African trypanosomiasis epidemiological settings requires fine-tuning control strategies to facilitate disease elimination. *Research and Reports in Tropical Medicine*, *4*, 1–6. https://doi.org/10.2147/RRTM.S40157

Sivajothi, S., Reddy, B. S., & Papazahariadou, M. G. (2016). Polypeptide Profiles of Diminazene Aceturate Resistant Trypanosoma evansi Organisms Isolated from a Buffalo. *J Veter Sci Med. J Veter Sci Med*, *4*(1).

Stuart, K., Brun, R., Croft, S., Fairlamb, A., Gürtler, R. E., McKerrow, J. & Tarleton, R. (2008). Kinetoplastids: related protozoan pathogens, different diseases. *Journal of Clinical Investigation*, *118*(4), 1301–1310. https://doi.org/10.1172/JCI33945

Thumbi, S. M., McOdimba, F. A., Mosi, R. O., & Jung'a, J. O. (2008). Comparative evaluation of three PCR base diagnostic assays for the detection of pathogenic trypanosomes in cattle blood. *Parasites & Vectors*, *1*(1), 46.

Torr, S. J., Maudlin, I., & Vale, G. A. (2007). Less is more: restricted application of insecticide to cattle to improve the cost and efficacy of tsetse control. *Medical and Veterinary Entomology*, *21*(1), 53–64.

Torreele, E., Bourdin Trunz, B., Tweats, D., Kaiser, M., Brun, R., Mazué, G. & Pécoul, B. (2010). Fexinidazole – A New Oral Nitroimidazole Drug Candidate Entering Clinical Development for the Treatment of Sleeping Sickness. *PLoS Neglected Tropical Diseases*, *4*(12), e923.

Tsegaye, B., Dagnachew, S., & Terefe, G. (2015). Review on Drug Resistant Animal Trypanosomes in Africa and Overseas. *African Journal of Basic & Applied Sciences*, *7*(2), 73–83. https://doi.org/10.5829/idosi.ajbas.2015.7.2.9370

Urech, K., Neumayr, A., & Blum, J. (2011). Sleeping sickness in travelers - do they really sleep? *PLoS Neglected Tropical Diseases*, *5*(11), e1358.

van Grinsven, K. W. A., Van Den Abbeele, J., Van den Bossche, P., van Hellemond, J. J., & Tielens, A. G. M. (2009). Adaptations in the glucose metabolism of procyclic Trypanosoma brucei isolates from tsetse flies and during differentiation of bloodstream forms. *Eukaryotic Cell*, *8*(8), 1307–11. https://doi.org/10.1128/EC.00091-09

Vreysen, M. J., Saleh, K. M., Ali, M. Y., Abdulla, A. M., Zhu, Z. R., Juma, K. G., & Feldmann, H. U. (2000). Glossina austeni (Diptera: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. *Journal of Economic Entomology*, *93*(1), 123–35.

Wamwiri, F. N., & Changasi, R. E. (2016). Tsetse Flies (*Glossina*) as Vectors of Human African Trypanosomiasis: A Review. *BioMed Research International*, *2016*, 1–8. https://doi.org/10.1155/2016/6201350.

Wang, Y., Utzinger, J., Saric, J., Li, J. V, Burckhardt, J., Dirnhofer, S., Holmes, E. (2008). Global metabolic responses of mice to Trypanosoma brucei brucei infection. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(16), 6127–32.

Wenzler, T., Boykin, D. W., Ismail, M. A., Hall, J. E., Tidwell, R. R., & Brun, R. (2009). New treatment option for second-stage African sleeping sickness: in vitro and in vivo efficacy of aza analogs of DB289. *Antimicrobial Agents and Chemotherapy*, *53*(10), 4185–92. https://doi.org/10.1128/AAC.00225-09

Wuyts, N., Chokesajjawatee, N., & Panyim, S. (1994). A simplified and highly sensitive detection of Trypanosoma evansi by DNA Amplification. *The Southeast Asian Journal of Tropical Medicine and Public Health*, *25*(2), 266–271.

Yarlett, N., & Bacchi, C. J. (1988). Effect of DL-alpha-difluoromethylornithine on methionine cycle intermediates in Trypanosoma brucei brucei. *Molecular and Biochemical Parasitology*, *27*(1), 1–10. https://doi.org/10.1016/0166-6851(88)90019-9

Yun, O., Priotto, G., Tong, J., Flevaud, L., & Chappuis, F. (2010). NECT Is Next: Implementing the New Drug Combination Therapy for Trypanosoma brucei gambiense Sleeping Sickness. *PLoS Neglected Tropical Diseases*, *4*(5), e720. https://doi.org/10.1371/journal.pntd.0000720

APPENDICES

APPENDIX 1: *In vitro* drug sensitivity set up in a 96-well microtiter plate

1 / A	2	3	4	5	6	7	8	9	10	11	12
B											Drug A Conc. 1
C											
D											Drug A Conc. 2
E											
F											Drug A Conc. 3
G											
H											

Drug sensitivity tests done in duplicate; Columns B&C assayed the same concentration of the test compound, etc. The outer wells were excluded from the tests.

APPENDIX 2: Primers used in the PCR amplification of isolates KETRI 2537 and KETRI 3867

Species/PCR	**Primers**	**Reference**
(KETRI 2537) SRA PCR	(F)SRA A: 5'GACAACAAGTACCTTGGCGC 3' (R) SRA E: 5'TACTGTTGTTGTACCGCCGC 3'	Gibson *et al.* 2002.
(KETRI 3867) TCF PCR	(F) 5'CGAGAACGGCACTTTGCGA 3' (R) 5'GGACAAACAAATCCCGCACA 3	Thumbi *et al.* 2008.
(KETRI 3867) TCK PCR	(F) 5'GCGGCAGGTCGACGGATC 3' (R) 5'CCCTCGAGAACGAGCA 3'	Thumbi *et al.* 2008.
(KETRI 3867) TCS PCR	(F) 5'CGAGAACGGGCACTTTGCGA3' (R) 5'GGACAAAGAAATCCCGCACA3'	Masiga *et al.* 1992.

APPENDIX 3: Letter of Approval – IACUC.

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Ref: C/TR/4/325/138

31st May 2013

Institutional Animal Care and Use Committee (IACUC)

Dear Dr. Monica Maichomo (PI) and Maureen Kamau (JKUAT, student),

Re: Evaluation of the safety and Efficacy of MG-192 as a Trypanocidal drug using invitro and invivo (mouse) models

This committee has received and reviewed your project proposal entitled "evaluation of the safety and Efficacy of MG-192 as a Trypanocidal drug using invitro and invivo (mouse) models" (C/TR/4/325/138). The committee is satisfied that the study protocols adequately meet the IACUC animal welfare standards in addition to other relevant guidelines provided by Kenya Veterinary Association. The committee therefore confirms that it will support the study. The committee further wishes to emphasize that during the project implementation phase, the responsibility for animal welfare lies with the investigators. This committee may make impromptu visits to ensure compliance with its regulations.

Yours sincerely,

Dr. J.M Mutuku
Coordinator, IACUC, TRC

APPENDIX 4: Publication

International Journal of Sciences: Basic and Applied Research (IJSBAR)

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Activity of MG-192 Against in Vitro Cultures of Infective Trypanosome Species and its Acute Toxicity Effects in Mice.

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