Investigating the Efficacy and Anti-Resistance Activity of Fexinidazole in Conjunction with Eflornithine Against Trypanosoma Brucei for Treatment of Human African Trypanosomiasis

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INVESTIGATING THE EFFICACY AND ANTI-RESISTANCE ACTIVITY OF FEXINIDAZOLE IN CONJUNCTION WITH EFLORNITHINE AGAINST TRYPANOSOMA BRUCEI FOR TREATMENT OF HUMAN AFRICAN TRYPANOSOMIASIS by Mercy Toma

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Human African Trypanosomiasis (HAT) is one of 17 neglected tropical diseases prioritized by the World Health Organization (WHO). Neglected tropical diseases are diseases which affect poor or developing countries and which do not get as much attention as the ‘big three’: malaria, tuberculosis and HIV/AIDS. HAT specifically affects countries in Sub-Saharan Africa and is caused by a parasitic protozoa, Trypanosoma brucei. There are two stages of HAT: the early haemolymphatic and late meningoencephalitic stages. There are also two subtypes of the disease caused by either T. b. gambiense or T. b. rhodesiense forms of the parasite. There are four drugs currently used to treat HAT, depending on the subtype of disease and whichever stage a patient is in. The nitroheterocyclic drug, Fexinidazole is currently in clinical trials for the treatment of both the early and late stages of HAT as well as the two subtypes. However, as with most antiparasitic drugs, potential parasitic resistance of the target parasite to Fexinidazole is an issue which must be taken into consideration and dealt with. Combination therapy is a method by which the likelihood of potential parasite resistance is reduced. The therapy uses two chemically unrelated drugs to treat a disease and is based on the theory that parasitic resistance to a combination therapy is less likely than resistance to a monotherapy. The combined drugs usually work additively or synergistically to treat the disease as well. Eflornithine (also known as α-difluoromethylornithine or DFMO) is a drug currently used in combination with nifurtimox to treat the second stage of HAT caused by T. b. gambiense. Nifurtimox and Fexinidazole are both nitroheterocyclic drugs and as a result, have similar modes of action. Therefore, I believe that eflornithine is the ideal partner drug to be used in conjunction with Fexinidazole since it has been shown to be effective in combination with a Fexinidazole-related drug (nifurtimox) but is not chemically related to Fexinidazole. The use of this combination therapy will allow prolonged use of Fexinidazole in treating HAT and contribute towards the eventual elimination of the disease.

Keywords: Human African Trypanosomiasis (HAT), sleeping sickness, haemolymphatic, meningoencephalitic, Trypanosoma brucei gambiense, Trypanosoma brucei rhodesiense, eflornithine, fexinidazole, combination therapy, monotherapy
Introduction

*Human African Trypanosomiasis (HAT)*

Human African Trypanosomiasis (HAT) is a neglected tropical disease which is communicable and prevails in tropical climates. The disease, also known as sleeping sickness, is caused by the parasitic protist *Trypanosoma brucei* and is transmitted from host to host by the tsetse fly (*Glossina* sp.)(1). The reason for the disease’s restriction to tropical climates is due to its vector, the tsetse fly, being prevalent only in such climates. The fly is specifically restricted to areas between the latitudes of 14°N and 20°S. as these areas have temperatures between 16°C–38°C and relative humidity levels of 50%-80%; temperatures ideal for tsetse fly survival(2). As a result, HAT is endemic to thirty-six countries in sub-saharan African countries which fall within the above-mentioned latitudes (shown in fig. 1)(3,4). These countries cover an area of 9 million km² leaving an estimated 60 million out of the 400 million inhabitants of these areas at risk of contracting the disease(4,5).

*Neglected Tropical Diseases*
Most diseases, such as HAT, which affect the poor and vulnerable in underdeveloped or developing countries have been grouped together under the title, “Neglected Tropical Diseases” (NTDs)(6). NTDs are different from the three major diseases- HIV/AIDS, tuberculosis and malaria- which receive a great deal of attention and funding though these three also affect developing countries(6–8). However, this does not mean that NTDs are completely ignored by the world. As a matter of fact, one-half of the 2015 Nobel Prize in Medicine was awarded to scientists William C. Campbell and Satoshi Omura for their work in developing avermectin, a novel therapy against onchocerciasis and lymphatic filariasis which are both caused by roundworm parasites and are both NTDs(9–11). The other half of the prize was awarded to Youyou Tu for discovering a new therapy against malaria(10). The awarding of the prize to the above-mentioned diseases, especially onchocerciasis, marked a milestone in infectious diseases affecting poor countries. The World Health Organization (WHO) has prioritized seventeen of these diseases, including HAT, which are caused by four major types of pathogens: protozoa, helminths, viruses and fungi(12).
pathogens thrive in tropical and sub-tropical climates, hence why they do not prevail in other countries but only prevail in the countries they do (as seen in fig. 2).

Together the seventeen NTDs affect more than a hundred countries and 1.4 billion people which is approximately one-sixth of the world’s population. In terms of mortality, the diseases cause the deaths of up to 500,000 people annually. The reason why the diseases thrive in poor areas is due to the fact that these areas suffer from inadequate access to clean water, poor nutrition, and poor hygiene amongst other less than optimal living conditions which are ideal for communicable diseases (13). In addition, the diseases leave those who are infected unable to work and earn a living due to cognitive and physical impairment. As a result, NTD infections result in a vicious cycle of poverty in infected communities where they are both its cause and effect (6). Although these diseases are termed “neglected,” commitment to controlling the various NTDs have increased internationally in recent years (7). In fact, in the year 2012, WHO formulated a “Roadmap” towards the control and the elimination of NTDs (7). For some diseases, the strategy for control was through preventive chemotherapy. For other diseases, such as Human African Trypanosomiasis (HAT), prophylactic treatment is not used; instead, the method of control is based on detection of the cases along with intensified disease management (IDM) (7). In this thesis, a literature review of the efforts that have been taken to control HAT so far will be provided. However, this cannot be done without some background information about the disease, something that will be discussed as well. In addition, a proposal will be made for the potential improvement of HAT treatment, especially if WHO’s plan target for the elimination of HAT by 2020 is to be achieved (7).
HAT History

Sleeping sickness was first associated with the slave trade in early modern times as the earliest accounts of the disease at that time were written by slave trade companies’ ship doctors and medical officers(14). The first accurate medical report of HAT was published in 1734 by an English naval surgeon named John Atkins. However, despite increasing reports of sleeping sickness in the nineteenth century, details about it were not known. It was not till the year 1852 that sleeping sickness was associated with the bite of a tsetse fly. This relationship was discovered by David Livingston, a Scottish missionary and explorer. Although it was now known how the disease was transmitted, the actual causative agent was still unknown.

Trypanosomes were not identified as the parasites responsible for HAT until 40-50 years after the discovery of its vector was made. Scottish pathologist and microbiologist, David Bruce discovered the protist, Trypanosoma brucei as the agent of infection in cattle trypanosomiasis (also known as cattle nagana) in 1895. Definitive identification of the trypanosoma parasite as the cause of HAT occurred when the protist was observed in the blood of an infected human in by a British Colonial surgeon in 1901. The following year, the parasite was also observed in the cerebrospinal fluid of infected patients.

There have been three major epidemics of Human African Trypanosomiasis that have been recorded and they all happened in the twentieth century(14,15). The first one was a severe epidemic that caused the deaths of between 300,000-500,000 people. It began in 1896 and did not end until 1906. It affected mainly Congo and Uganda, but also Kenya. This epidemic occurred during colonial times and it curbed the development of colonial territories(5). This first epidemic also began before any information had been gathered about sleeping sickness.
Therefore, all the discoveries concerning HAT which were described above happened as a result of the colonial administration’s attempts to prevent the disease from dismantling their colonies and leaving them without laborers(5,14). Once the disease had been clearly identified, along with its causative agent, control measures were established to stop the HAT epidemic.

The second major HAT epidemic began in 1920 and did not die down until the 1940s. The discovery of two drugs for treating HAT, suramin, which is still used today, and tryparsamide, an organo-arsenical drug, contributed greatly to the fight against the second epidemic. Other additional measures such as the introduction of mobile teams were undertaken to control the spread of the disease. With the mobile teams, systematic detection and treatment of infected people was initiated. Other measures which were also introduced and contributed to the control of the epidemic, including vector control and host reservoir control(14).

The discovery of more drugs against HAT such as melarsoprol and pentamidine along with the combination of the systematic case screening and vector control eventually led to a dramatic decrease in HAT incidence once the 1960s began(14). In fact, HAT transmission in endemic areas was disrupted at this time so much that interest was lost in the disease(5). A loss of interest in the diseases resulted in discontinued surveillance and the risk of re-emergence of the disease was not taken into consideration(5). Discontinued surveillance was encouraged by HAT-endemic countries gaining their independence from the colonial powers in the 1960s. This led to both political and economic instability as the nations tried to determine how to stand on their own feet. The economic instability also negatively impacted the healthcare infrastructure in these countries so that HAT surveillance and control was no longer a priority(14).
The combination of reduced awareness of HAT, diminished HAT priority, and social instability and conflicts made it difficult for control interventions, resulting in a re-emergence of the disease in epidemic proportions(2). This constituted the third major HAT epidemic, lasting from the 1980s to the 1990s. The only countries which were affected were Angola, the Democratic Republic of the Congo, Southern Sudan, and the West Nile district of Uganda; but, only a fraction of these areas were under surveillance(2,14). An estimated 30,000 cases were reported annually during this time. However, due to the fact that civil wars and social upheaval prevented access to a large number of the affected populations, WHO estimated that the number of people affected was more likely 10 times more than the reported number of cases(5). As a result, WHO improved its coordinating abilities and began to advocate and raise awareness of the disease so that both the public and private sectors contributed resources for HAT control and surveillance(2,5).

Eventually, all the above-mentioned efforts began to yield results; the number of HAT cases began to decrease steadily, falling below 10,000 since 2009. Figure 3 below shows the rise and fall in the incidence of cases, including during the period when HAT reached epidemic proportions. Although global incidence of HAT is low and seems to be declining, it is important to note that there are still areas in Sub-Saharan Africa where it remains a “hidden epidemic” under continuous surveillance(2,16). Therefore, the disease should not be written off as a public concern as this would be premature(16). Additionally, history has shown that HAT incidence is characterized by epidemics that occur in episodes and resurgences, indicating the importance of maintaining HAT control methods(17).
HAT pathogen

Sleeping sickness, as mentioned before, is caused by *Trypanosoma brucei*, a unicellular, flagellated protozoan parasite (seen in fig. 4). The flagella helps to move the parasite in whichever direction it elongates its body (16). With the flagella, the trypanosome is able to travel at speeds up to 20 um s\(^{-1}\), making it a highly motile cell (18). There are two subspecies of *T. brucei* which infect humans, *T. b gambiense* and *T. b rhodesiense* (19). The gambiense form of the disease is known to be the causative agent of HAT in West and Central Africa while the rhodesiense form infects the eastern part of
Africa (20). The gambiense form is also more chronic, lasting up to three years and accounting for approximately 98% of observed HAT cases (20). This subtype of HAT is anthroponotic, meaning it primarily affects humans but can also be transferred to animals (2,21). It is transferred from host to host by riverine tsetse flies, *Glossina palpalis* (22,23).

Conversely, the rhodesiense form of HAT is a more acute and progressive type of the disease, lasting only months rather than years (2). This is the main difference between both forms of the disease. Another difference between the two is that the rhodensiense form, unlike the gambiense one, is a zoonotic disease (22). Its transmission cycle happens mainly between wild and domestic animals but can also intensify to human infection (2,20). The tsetse flies responsible for transmission of this subtype of *T. brucei* are the Savannah flies (*Glossina morsitans*) (22,23). The rhodesiense form of HAT accounts for the other 2% of observed HAT cases (2).

There is a third subtype of *T. brucei*, *T. b. brucei*. Unlike the other subtypes, this one does not infect humans but is only infectious to wild and domestic animals (14,24). This subtype is commonly used in the models for experiments dealing with HAT (2). *T. b. brucei*, like the other subtypes, is also transmitted by the tsetse fly. The three subtypes are morphologically indistinguishable from each other and also have similar life cycles (25).

*T. brucei* life cycle in the tsetse fly

Despite the fact that HAT is highly life-threatening, the causative pathogen cannot infect a new host if it is not done through the bite of a tsetse fly (*Glossina spp.*) (16). However, the environment encountered by *T. brucei* during mammalian infection differs from the
environment in the tsetse fly vector(17). Therefore, in order for the unicellular eukaryote to survive and proliferate, it must adapt by undergoing developmental changes throughout its life cycle in the different environments(25,26). These developmental transformations include changes in cell length and width, length of the flagella, and the relative position of the nucleus(16). As a result, the changes result in the formation of different *T.brucei* forms with morphological differences(26).

Tsetse flies are blood-feeding insects which obtain energy and nutrients solely from the blood that they feed upon(27). As a result, they are referred to as hematophagous arthropods(28). The insects pick-up the *T. brucei* parasite when they feed on the blood of an infected host(29,30). A picture of the tsetse flying biting a mammal is shown in fig. 5. The life cycle of the parasite while is in the insect is known as the procyclic stage(31). The tsetse fly ingests *T.brucei* (into its midgut) from the bloodstream of the infected mammal while it is in its trypomastigote form. Two types of the bloodstream form (BSF) trypomastigotes are ingested by the fly during blood-feeding: the short stumpy (ST) form which cannot divide and the long slender (LS) form(16,32,33). Therefore, once in the insect’s midgut, ST trypomastigotes transform into procyclic trypomastigotes which are able to proliferate in the tsetse fly’s midgut, thereby, allowing for infection of the fly(34). LS trypomastigotes, however, are unable to transform and therefore, die out(16). It is thought that the ST forms are pre-adapted to the environment in the midgut which is why they are able to differentiate, unlike the LS.
forms(32,35). Figure 6 shows scanning electron micrographs of the BSF versus the procyclic form of *T. brucei*(17).

The procyclic form of the trypanosome is only infective of the tsetse fly and cannot infect mammals but must first develop through a series of transformations (which will be discussed shortly) before it can do so(18,36,37). Once differentiation into the procyclic form of the trypanosome has occurred, multiplication of the cell begins(20). Infection of the fly’s midgut is not always successful as immunity-related factors subject proliferation to a series of bottlenecks(16). Infection is deemed successful if trypanosomes are found in the ectoperitrophic space (part of the midgut) three days post-infection as differentiation and proliferation lasts two to three days(16). After the infection of the tsetse fly has been established, the parasites then travel towards the anterior end of its alimentary canal (or midgut) to the proventriculus (PV). This journey is accompanied by elongation of the procyclic trypomastigotes into mesocyclic trypomastigotes(16,20). The mesocyclic trypomastigotes have longer flagella which help to enhance migration of the cells(16). Once the mesocyclic cells arrive at the proventriculus, they proceed to transform into the thinner and longer epimastigote forms of the *T. brucei* cell(20). Asymmetrical division of the epimastigotes occurs after the transformation which results in long and short epimastigotes. The long epimastigotes have more motility than their short counterparts and so serve as transport vehicles for them as they travel towards the salivary gland of the fly(16,20). Movement from the proventriculus to the
salivary gland is done via the fly’s foregut and proboscis, meaning only a small number of parasites are able to complete the journey(20). Therefore, the migration process serves as another bottleneck for *T. brucei* parasites in the tsetse fly(16). The cells that are able to circumvent this bottleneck then attach to the epithelium of the salivary glands via their flagella(16). After attachment, the epimastigotes, like the procyclic trypomastigotes, begin to proliferate.

Colonization of the tsetse fly’s salivary glands is achieved through normal cell division of the trypanosome. This is eventually followed by another round of asymmetrical division which results in the formation of a daughter cell that matures into a metacyclic form of the parasitic eukaryote. This metacyclic form is non-dividing and primed to adapt to or survive the environment in the mammalian host(34). Therefore, it is an infective form which can then be injected by the tsetse fly into a new host during feeding, thereby infecting it(16,30). However, maturation of the metacyclic trypomastigote is not complete until the cell acquired its variant surface glycoprotein (VSG) coat(18). It is the VSG-coated metacyclic trypanosomes that are ultimately released through the tsetse fly’s saliva(18). Genetic exchange, the mating of *T. brucei* cells, is said to happen in the salivary glands of the fly(24,38). The entire process, from ingestion of the BSF trypomastigotes to maturation of the metacyclic trypomastigotes takes about twenty to thirty days in the tsetse fly(36). This ability of the tsetse fly to acquire the trypanosomal parasite, allow it to mature and finally transmit to a new mammalian host is described as vector competence(37). A summary of the life cycle of the trypanosome in the tsetse fly is shown in figure 7 below(16).
Figure 3. Life cycle of T. brucei in the tsetse fly. (I) The fly ingests the long slender (LS) and short stumpy (ST) trypomastigote forms which are then present in its midgut. The ST forms differentiate into the procyclic trypomastigote forms which are proliferative in nature (II). After infection is established, the PC trypomastigotes migrate toward the anterior portion of the midgut, elongating into mesocyclic trypomastigotes (MS), which are found in the foregut (FG), in the process (III). Once the MS forms arrive at the proventriculus (PV), they differentiate into epimastigotes which then divide (DE) asymmetrically into long and short epimastigotes (IV). Long and short epimastigotes travel to the salivary glands and a fraction are able to attach themselves to the epithelium (AE). The epimastigotes then undergo normal cell division (Epi-Epi) in order to establish infection in the SG or asymmetrical division (Epi-Trypo) to produce infective metacyclic trypomastigotes (MT) (V, VI) (16).

Life cycle of T. brucei in mammalian hosts

In mammalian hosts, infection is initiated when an infected tsetse fly deposits metacyclic trypomastigotes in the connective tissue of the host’s dermal layer (28). Once it is deposited, the parasite proliferates at the site of inoculation before migrating to the lymph and blood circulation circuits (3). This is known as the early haemolymphatic stage of infection (18). Although it may cross through the walls of the lymphatic and blood capillaries into connective tissue, the parasite always remains extracellular (28). Eventually, the protozoan cells cross into the central nervous system (CNS) and cerebrospinal fluid by crossing the blood-brain barrier (BBB) or blood-CSF barrier (28). This is referred to as the late, meningo-encephalitic stage of
*T. brucei* infection(18). HAT is always fatal if left untreated due to this ability of the parasite to invade the CNS(18). The migration of the parasitic cells into the CNS provides a privileged environment which is not easily accessible by chemotherapy. Therefore, the CNS can serve as a reservoir, allowing *T. brucei* cells to re-infect the host(18).

Unlike its life cycle in the tsetse fly, during which the trypanosome is characterized by different forms, the lifecycle of the protozoan in its mammalian host consists mainly of two forms: long slender (LS) and short stumpy (ST) form of trypomastigotes(28). The two trypomastigote forms are easily distinguishable from each other under the microscope(26). The LS form is the dividing form while the ST form is irreversibly arrested in the cell cycle making it non-proliferative(25,39). The ST trypomastigote, however, is adapted for transmission into the tsetse fly as mentioned before(33,40). *T. brucei* also has transitional forms between the LS and ST trypomastigote forms. These forms are referred to as intermediate forms(32).

Transformation of the parasitic eukaryote from LS into ST forms occurs when parasite numbers increase in the host(41). The reason for this transformation is to maintain proliferation at sub-lethal levels in the host. In this way, the parasites avoid killing the host, as this would result in their death as well(39). Therefore, a balance always exists between the host and the parasite. *T. brucei* levels must be obtained at levels high enough for chronic infection to occur but must not be high enough to overwhelm the host before transmission occurs as this would lead to its extinction(39). The LS trypomastigotes have a density-sensing mechanism which allows them to detect when parasitic levels are getting lethal. This causes the arrest of some LS forms in a certain stage of the cell cycle (the G0/G1 stage), forming ST trypomastigotes which have a lifespan of about two to three days after the cell cycle arrest(39). In this way, host survival
is prolonged and transmission is also ready to be carried out (32). As a result, parasitemia in the host is marked by cyclical waves of rising and decreasing levels of infection (36) (shown in fig. 8).

The VSG coat and antigenic variation

As previously mentioned, *T. brucei* remains extracellular throughout its infection of a mammalian host. As a result, the parasite is left constantly exposed and vulnerable to the immune system of the host (42). Despite this, it is still able to establish prolonged infection in the host, resulting in a variety of pathological manifestations (43). The trypanosome is able to
do so because its surface is completely covered by a homogenous protein coat known as the variant surface glycoprotein coat (VSG)(30). This coat is comprised of approximately $10^7$ densely packed molecules of a single VSG type and is responsible for determining the phenotype of the parasite’s antigens(44).

The dense VSG coat shields the non-changing antigens on the surface of the protozoan parasite, preventing them from being detected or accessed by the adaptive immunity of the host(42,43). The VSG coat is also responsible for preventing *T. brucei* from activating the alternative complement pathway in the host(30). Despite this, the host’s immune system is still able to produce antibodies against the VSG, eventually resulting in lysis of the parasitic cells. In order to circumvent this and continue to survive, *T. brucei* periodically switches to an immunologically different VSG(42). This ability of the trypanosome to change the identity of the VSG type comprising its glycoprotein coat is referred to as antigenic variation(42). The trypanosome has up to 2000 genes responsible for the expression of the VSG types. In addition, genetic recombination of these genes also account for distinct VSG types. The parasite possesses mechanisms which ensure that only one VSG gene is transcribed at a time(28). As a result, the trypanosome has an almost inexhaustible repertoire of antigens that can be expressed on its coat(44,45). Experimentally, a single trypanosome was observed to express at least 100 distinct VSG coats. However, this is an underestimation due to limited ability to detect the varying VSG types(30).

Antigenic variation is one of the most remarkable mechanisms of adaptation that the African trypanosome exhibits(28). It is also the most important contributing factor to the parasite’s ability to evade the host’s immune system and establish chronic infection(28,44).
During trypanosomal infection, a few parasites switch their VSG type at random(39,45). Therefore, when the immune system recognizes and clears parasites exhibiting the previous VSG variant, the parasites with the new variant survive. It is usually the short stumpy (ST) trypomastigote forms that are the survivors as they are more resistant to antibody-dependent complement-mediated cell clearance than their long slender counterparts(46). These survivors eventually give rise to a peak in parasitemic levels which is again decimated when antibodies are produced against those variants(42). As a result, infection of the host is characterized by cyclical waves of parasitemia (seen in fig. 9)(36,42,45). This eventually results in exhaustion of the host’s immune system(28). The ability of the trypanosome to undergo antigenic variation has been a major obstacle in vaccine development against HAT and has made prophylactic treatment an unlikely option in HAT control(47,48). This is evidenced by the fact that no vaccine trial with promising results has led to positive field trials(49).

The VSG coat is only found on the bloodstream form (BSF) long slender and short stumpy trypomastigotes. When the BSF trypomastigotes differentiate into the procyclic trypomastigote in the midgut of the tsetse fly, they shed the dense VSG coat and replace it with
a procyclin coat (25, 41). As a result, if the parasite were ever to transform into its procyclic form within the mammalian host, it would be rapidly cleared by the immune system as it does not have sufficient protection. Considering this information, induction of premature transformation of *T. brucei* into its procyclic form while in the mammalian host could serve as a potential chemotherapeutic approach for treating HAT (25).

**Clinical presentation: signs, symptoms and diagnosis**

It was mentioned previously that during HAT infection, the *T. brucei* parasite progresses through two stages of infection. The first and early stage is referred to as the haemolymphatic stage. It is the stage of infection during which the parasite is restricted to and proliferates in the blood and lymph of the host (50). Eventually, the parasite invades the CNS by crossing the blood-brain barrier (BBB) or blood-csf barrier (BCB) (51). This constitutes the second or late stage of HAT infection, also referred to as the meningo-encephalitic stage (52, 53). Infection always progresses from the first stage to the second stage if left untreated as the stages are sequential in nature (52).

The progression of HAT from the early to the late stages is characteristic of infection by either of the two *T. brucei* subtypes, *T. b. rhodesiense* and *T. b. gambiense* (3). However, as discussed previously, rhodesiense infection is the more acute form of HAT, lasting only months (54). On the other hand, HAT caused by the gambiense form presents with a more chronic form, lasting years (1, 3). As a result, invasion of the CNS, progression to the second stage of infection, occurs after about 3 weeks in rhodesiense HAT infection and after about a
year in gambiense HAT(55,56). This constitutes the main difference between the two HAT infection subtypes.

HAT infection by the rhodesiense subtype of the trypanosome is marked by a chancre in 5-26% of infected patients(1,50). The chancre develops as a result of an inflammatory response to the inoculation of *T.b rhodesiense* parasites into the patient through the bite of the tsetse fly. The chancre marks the area where the patient was bitten by the fly and is the first sign of disease in these cases(1,50). The chancre is characterized by local redness, swelling, heat, and tenderness (as seen in fig. 10) at the site of the bite(1). The chancre is rarely seen in patients infected with the gambiense form of HAT.

Due to the differences in infected areas between the two stages of sleeping sickness, they present differently in clinical situations. The early stage of HAT presents with non-specific symptoms such as intermittent fever, headaches, severe itching of the skin, skin lesions, weakness, anemia, lymphadenopathies, endocrine disturbances, muscoskeletal pains, cardiac disorders, hepatosplenomegaly, and edema of the face and the extremities(1–3). Once the disease progresses to the second stage, it presents clinically with features that can be categorized into psychiatric, motor and sensor abnormalities, and sleep disturbances(57). While these signs and symptoms are more specific than those of early HAT stage infection, they are still not individually diagnostic of HAT, as some of them characterize other CNS diseases as
well(57). If left untreated, HAT is fatal to the infected patient as it progresses to systemic organ failure, cerebral edema, coma, and death(3,57).

Diagnosis of HAT is achieved using a combination of clinical and investigative data(57). If the set of symptoms described above present clinically in the context of a geographical location endemic to HAT, this would serve as an important clue for diagnosis(57). However, even in these endemic areas, rhodesiense HAT, for example, which is characterized by fevers of 103-105°F(54), is often misdiagnosed as other febrile endemic diseases with similar clinical signs(58). Examples of these diseases include malaria, enteric fever, meningitis, tuberculosis, and HIV/AIDS(58). Therefore, the non-specificity of the disease’s signs and symptoms requires that diagnosis be confirmed by lab tests and results(52,58). A confirming lab result would be the detection of trypanosomes in the patient’s blood (seen in figure 11) or other tissues such as the lymph node during the early stage of the disease(57).

Parasites are relatively easy to detect in rhodesiense HAT due to the presence of numerous bloodstream trypanosomes in this subtype of the infection(57,58). However, this is not the case in infections caused by *T. b gambiense* as fluctuating parasitemic levels, since cyclic parasitemia (as a result of antigenic variation discussed previously) is a characteristic of this infection subtype(57,58). Therefore, parasitological confirmation is more difficult with
gambiense HAT. This is where serological tests such as the one currently being used, card agglutination test for trypanosomiasis (CATT), come in. CATT is a simple and rapid direct agglutination assay used to detect the presence of antibodies specific to *T. b. gambiense* in the blood, plasma or serum of patients(59). The assay has a sensitivity rate of 87-98% and a specificity rate of 93-95% making it very reliable(21). CATT is used for screening purposes in the field, followed by parasitological confirmation(59). In other words, once a patient yields a positive CATT result, a microscopic search is then done for trypanosomes in the lymph nodes and blood(52).

An important part of HAT diagnosis is determining which stage of infection a HAT patient is in as therapeutic decisions are based on this information. The haemolymphatic stage must be reliably distinguished from the meningo-encephalitic stage as failure to treat a patient with *T. brucei* CNS infection results in fatality. Conversely, inappropriate treatment of an early stage patient for CNS infection involves high risk of unnecessary drug toxicity(57). Reliable staging of HAT is done by performing a lumbar puncture on the patient in order to examine the CSF(52,55). The criteria for CNS involvement in HAT infection is defined by WHO as the detection of trypanosomes in the CSF or a white blood cell count (WBC) of >5 cells/µl, or both (3,52,57).

**Current treatments**

The BBB is highly selective – it prevents 98% of all known compounds from entering into the CNS including drugs used in treating HAT(60). As a result, drugs used in the treatment of late stage sleeping sickness are toxic in their own right and differ from those used in treating
the early stages (3,60). The chemotherapeutic agent used in treating HAT also depends on which subtype of the parasite is the causative agent of infection as different drugs are used to treat the different HAT subtypes (3).

First stage sleeping sickness is treated with suramin in rhodesiense infection and pentamidine in the gambiense subtype (61). Suramin is a trypanocidal drug which is a derivative of a naphthalene urea compound (seen in fig. 12) (62). The drug is administered intravenously over the course of thirty days with a complex dose regimen (1,50). It is associated with complications such as renal failure, skin lesions, nephrotoxicity bone marrow toxicity, and peripheral neuropathy (1,50). However, these side effects are usually mild and reversible (1). Anaphylactic shock is another side effect of suramin treatment when acute hypersensitivity reactions occur. As a result, a low test dose is usually applied to the patient before the beginning of treatment (1).

Pentamidine, an aromatic diamidine (seen in fig. 13), has been in use for the treatment of HAT infection since the 1930s (15). The drug was originally used with the intention of starving bloodstream trypanosomes of glucose; however, it was later discovered that it was trypanocidal in its own right (15). It is administered once daily over the
course of seven days though intramuscular means (3, 15). The side effects of pentamidine treatment include nausea and vomiting, hypoglycemia, and pain at the site of injection (1, 3). Despite this, the drug is generally well-tolerated (3). Treatment has proven to be effective and able to prevent the progression of disease (57).

The treatments used in combatting second stage HAT are melarsoprol, eflornithine, and a combination therapy of eflornithine and nifurtimox (NECT) (3). Melarsoprol is a trivalent organic arsenical compound (seen in fig. 14) that was first discovered to treat second stage HAT in 1949 (61). Its mechanism of action is not known (52). However, the drug was found to be effective in treating both the gambiense and rhodesiense forms of sleeping sickness and was the only available drug for treating both for fifty years after it was first discovered (1). Its administration involves three to four doses daily over the course of three to four weeks followed by clinical evaluation every six months during the next two years for follow-up purposes (57). This treatment course has been shortened to daily injections of the drug for ten consecutive days (1, 61). However, melarsoprol as an arsenic-based drug is toxic and is known to cause reactive encephalopathy in about 10% of treated patients (50). Reactive encephalopathy is characterized by coma and repeated convulsions which result in the death of 50% of these patients (52). Due to the toxicity and side effects of melarsoprol, a search was undertaken to find an alternative treatment for late stage HAT infection. Melarsoprol has a high efficacy rate in HAT patients but has been
observed to have a failure rate of about 30% (57). This treatment failure is possibly caused by drug resistance in trypanosome cells (15).

Eventually, in the mid-1980s, eflornithine (α-difluoromethylornithine or DFMO seen in fig. 15) was found as an alternative treatment and safer treatment for late stage sleeping sickness (52). In 2000, twenty years after its efficacy was first established, it was made available for clinical use (1). The drug functions by affecting the synthesis of polyamines - important organic compounds required for growth and multiplication by all eukaryotic cells - in trypanosomal cells (52). Specifically, eflonithine irreversibly inhibits trypanosomal ornithine decarboxylase (ODC), an enzyme involved in the metabolic pathway.

Eflornithine chemotherapy involves the administration of fifty-six intravenous injections of the drug over the course of fourteen days: 100mg/kg two hour infusions four times daily (52,63). Once it was made available for clinical use, eflornithine was made the first-line treatment for treatment of gambiense HAT (1,52). However, its use is constrained by the fact that its administration is long and burdensome and not ideal for HAT-infected areas where material and human resources are already limited (1,63). To mitigate some of these difficulties, WHO provided kits with the necessary supporting materials for treatment and coordinated training of personnel from national sleeping sickness control programs (1,52). The WHO made these efforts in 2006. Following this, eflornithine use as first-line treatment for gambiense sleeping sickness began to increase (52).
Nifurtimox is a nitrofuran derivative organic compound (seen in fig.16). The drug is used to treat Chagas disease caused by *Trypanosoma cruzi* but was found to work as a partner drug with eflornithine in a combination therapy\(^{(52,62)}\). Nifurtimox and eflornithine combination therapy (NECT) was implemented in the treatment of sleeping sickness in an effort to shorten and simplify eflornithine monotherapy\(^{(1)}\). The combination therapy was discovered to be less cumbersome and difficult to administer, requiring only fourteen intravenous injections of eflornithine over seven days rather than the fifty-six injections of eflornithine monotherapy over fourteen days\(^{(52,63)}\). Therefore, NECT not only involves a fourfold decrease in the number of IV (intravenous) infusions of eflornithine needed but also a decrease in the duration of treatment. Nifurtimox is taken orally three times per day for the duration of ten days\(^{(63)}\).

NECT also involves a decrease in cost of treatment and an increase in the efficacy of eflornithine\(^{(3,63)}\). Therefore, NECT has been recommended by WHO as the first-line treatment for late stage HAT infection by *T.b gambiense*. In 2009, it was added to WHO’s List of Essential Medicines\(^{(1,61)}\). The addition of the combination therapy to this list was what opened the way for its use in HAT-affected countries\(^{(63)}\). The discovery of the combination therapy was a breakthrough in HAT treatment as it was the first new registered HAT drug since 1981\(^{(57)}\). However, melarsoprol still remains the only treatment for the second stage of rhodesiense sleeping sickness\(^{(1)}\).
Vector control

One other way through which HAT is controlled, apart from chemotherapy, is vector control. By controlling the vector, transmission of HAT is reduced and people are protected (64). One of the methods of vector control uses insecticide-treated and non-treated fly traps or screens which allow for a reduction in fly density in areas where it is high (1,4). Some of the traps are color-baited as it was discovered that the flies are drawn to blue/black colors (27). Other available control methods which are used include insecticide-treated cattle, aerial or ground spraying of low dose insecticides (such as pyrethroids) and the fogging of tsetse fly resting sites (21,65,66).

Interestingly, vector control is also achieved through the introduction of sterile tsetse males into areas populated with the insects. This technique is referred to as SIT, sterile insect technique (21). The sterile males mate with the females but do not produce offspring. However, tsetse females can only mate once during their lifetime. Therefore, they themselves are practically rendered sterile as well and no new tsetse flies are produced (21,23). Considering that rhodesiense HAT is zoonotic in nature, control of the animal reservoir is the main challenge. For example, cattle are known to be major reservoirs of the disease (67). However, cattle can be treated with insecticides (a cost-effective method of vector control), but control of the disease and vector in wildlife living in game parks and protected areas is difficult (1). In the absence of prophylactic treatments against sleeping sickness, vector control is very important and research continues to be done to improve control techniques (64).
Fexinidazole

The harmful side effects of current HAT treatments, especially melarsoprol, the only treatment for late stage HAT, along with the report of treatment failures led to the search for new drugs against HAT(68). Research was performed to find not only new HAT drugs but also effective and safer ones as well(3). The newly discovered drug would also be ideal if it could treat both the haemolymphatic and meningo-encephalitic stages of sleeping sickness as this would preclude staging of the disease and its associated difficulties(57).

Fexinidazole (1H-imidazole,1-methyl-2-[[4-methylthio] phenoxy] methyl] 5-nitroimidazole), a 2-substituted 5-imidazole organic compound, is a HAT drug that is in phase II/III clinical trials for use against HAT(3,69). The chemical structure of the drug can be seen in fig. 17(69). It belongs to the nitroimidazole group of drugs which in turn belong to the nitroheterocyclic family of drugs. Nitrofurans such as nifurtimox belong to the nitroheterocyclic group of drugs as well. Nitroheterocyclic drugs all have the same general mode of action(70). In fact, it was the success reports of nifurtimox used in combination with eflornithine which led to a renewed interest in nitro-based drugs as treatment for infectious diseases(71). This resulted in the rediscovery of Fexinidazole and its efficacy in treating HAT by the Drugs for Neglected Diseases initiative (DNDi)(69). The drug was actually first synthesized in 1978 and showed promise, along with its primary metabolites (shown in fig. 17), as treatment against organisms...
such as *Trypanosoma cruzi* and *Entamoeba histolytica* (72). However, development of the drug was not followed up after preclinical studies. DNDi rediscovered it while searching for old and new imidazoles with activity against *T. brucei* (72). Pharmacological assessment of fexinidazole in mouse models showed that the drug is effective in treating both the haemolymphatic and meningo-encephalitic stages of sleeping sickness. Additionally, *in vivo* and *in vitro* studies have demonstrated that it exhibits activity against both *T. brucei gambiense* and *T. b rhodesiense* (73). Therefore, Fexinidazole has shown promise as the ideal drug to be used against HAT.

**Combination therapy**

The use of NECT against HAT and its breakthrough in the treatment of the disease exhibit the advantage of combination therapies. Drug combination therapies often used to result in increased efficacy, decreased toxicity, and delayed onset of drug resistance in the target parasites (57). Emergence of parasitic resistance is one of the major drawbacks of long-term monotherapies, hence why combination therapies are used (63). The reasoning behind the use of combination therapies to combat or decrease potential resistance is that the likelihood of parasites developing resistance to two compounds is much lower than the likelihood of resistance to just one compound (71). Therefore, for the combination therapy to reduce the likelihood of resistance, the two drugs should be chemically unrelated, making cross-resistance between the two unlikely (74). If the two drugs are chemically unrelated, then they likely have different modes of action and can contribute to the decreased resistance potential. This is supported by the demonstration of cross-resistance between nifurtimox and fexinidazole which are both nitroheterocyclic drugs (70). The cross-resistance study showed that nifurtimox-resistant *T. brucei* cells exhibited relative resistance to fexinidazole as well. This phenomenon
was also observed between fexinidazole-resistant trypanosomes and nifurtimox treatment (71). However, nifurtimox-resistant cells were still sensitive to pentamidine and eflornithine, both chemically unrelated drugs (71). Ideally, partner drugs for new antitrypanosomal drugs should be found before widespread clinical use of the new drug.

The exact mechanism of action of nitroheterocyclic drugs such as fexinidazole is not known (70). However, they are believed to be prodrugs which, when reduced, cause damage to DNA, proteins, and lipids (71). They do this by relying on nitroreductase enzymes (NTR) to catalyze their reduction, producing cytotoxic species such as the superoxide anion and the hydroxyl radical (71,72). This mode of action is different from that of eflornithine which inhibits an enzyme involved in polyamine synthesis. Therefore, considering all the information presented on the advantage of combination therapies, it is my hypothesis that eflornithine will be the ideal partner drug for fexinidazole.

**Proposed experiment**

Eflornithine’s ability to serve as the partner drug for Fexinidazole will be determined by testing the efficacy of the combination therapy against *T. brucei* in comparison to the efficacy of Fexinidazole monotherapy. Before this is done, however, the optimal concentration of eflornithine to be used in conjunction of Fexinidazole must be found. In addition, the combination therapy will be tested against lab-generated fexinidazole-resistant (FxR) *T. brucei* to determine sensitivity of the trypanosomes to the drug combination.

**Methods and tools**

*Cell line and culture condition:*
The *T.b brucei* S427 bloodstream form will be used in all experiments. The cells will be cultured according to the protocol of Sokolova et al(71). The cells will be cultures at 37°C in HMI9-T medium. The medium will be supplemented with 2.5 μg/ml G418 according to the protocol. Fexinidazole-resistant (FxR) cells will also be cultured in these conditions.

**Determination of optimal eflornithine dose in vitro:**

This growth inhibition assay will be done according to the protocol used by Kaiser et al(74). Five different concentrations of eflornithine will be used in conjunction with 200mg of Fexinidazole. The different combinations will be introduced into 96-well plates along with the culture medium. Two additional plates will be used, one with no drugs added and another with 200mg of Fexinidazole only. The wells will then be inoculated with 2000 trypanosomes. Incubation of the cell cultures will be done at 37°C under a humidified 5% CO₂ atmosphere for 70h. Trypanosome growth inhibition caused by the different drug combinations and monotherapy will be analyzed using a microplate fluorescence scanner. The eflornithine concentration which is most effective in combination with fexinidazole will be used in subsequent assays.

**Determination of combination therapy efficacy:**

This part of the experiment will be done *in vivo* using three groups of adult NMRI male mice which will be fed and kept under standard conditions. Each group will contain ten mice for the purpose of this experiment. Infection of the mice with wild type (WT) *T.brucei* trypanosomes will be done by inoculating them (intraperitoneally) with $10^4$ of the parasite in 0.2mL of HM19-T medium(74). Forty-eight hours post-infection, one group will be injected with
Fexinidazole (200mg/kg), one will be injected with the combination of fexinidazole and eflornithine, and the last group will be left untreated. This group will serve as the control. The treatment will be repeated for three days. Mice will be monitored over a period of thirty days both for clinical signs of infection and parasitemia levels. Clinical signs include raised hair coats, dullness, excessive sweating and decreased appetite which normally coincide with a peak in parasitemic levels(75). Parasitemia levels will be determined using a hemocytometer on wet blood smears extracted from the tail. The blood smears will be examined microscopically as well.

*Generation of Fexinidazole-resistant (FxR) cells:*

Fexinidazole-resistant *T.brucei* cells will be generated *in vitro* according to the protocol described by Wyllie et al(70). FxR cells will be generated by exposing the parasitic cells to the continuous presence of Fexinidazole in culture. The cells will be subcultured in media with drug concentration increasing in a step-wise manner starting with a sublethal concentration of 1.0 μM until they are surviving and growing in 50 μM of Fexinidazole. Cloning of the now resistant cells will be done after 140 days of the cells in culture. Cloning will be achieved in the absence of Fexinidazole using limited dilution techniques. The cloned cell lines will then be tested for resistance to fexinidazole and the cell line displaying the most resistance will be used for subsequent experiments.

*Determination of combination therapy efficacy against FxR cells:*

This part of the experiment will be set up like the other in vivo study described above. However, mice will be inoculated with FxR cells rather than WT cells. Parasitemia levels will also be determined the same way.

**Proposed results**

If eflornithine eventually proves to be the ideal partner for the combination therapy, first, one of the three doses of eflornithine tested in conjunction with Fexinidazole will prove to be most effective. In other words, the drug combination involving this optimal dose will show a faster rate of activity against trypanosomes than the monotherapy and the other drug combinations. Once this optimal dose is determined, the efficacy of the monotherapy will then be compared to that of the monotherapy in vivo. Decreased parasitemic levels in comparison with the monotherapy should also be observed in this experiment.

Trypanosome resistance to Fexinidazole will be induced using the experimental protocol described above. The resulting *T. brucei* cell line will then be cloned as described in the experimental protocol. The cloned cell line that displays the most resistance to Fexinidazole will then be used for further testing. *In vivo*, this cloned cell line should be more sensitive to the eflornithine and Fexinidazole combination therapy than the monotherapy. In other words, parasitemic levels in FxR-infected mice should be lower when treated with the combination therapy in comparison to the mice treated with the monotherapy.

With combination therapies, there are two possible effects that can be observed in the results, synergistic or additive. A synergistic effect will yield results showing that the combination therapy was more effective, displaying lowered parasitemic levels over a shorter
time period than fexinidazole monotherapy. Conversely, an additive effect will yield results which show that the combination therapy is not inferior to the monotherapy alone, proving it is just as effective as clearing parasitemia as the monotherapy. It is difficult to predict which combined effect the two drugs will have when used in combination without the actual experiment being carried out.

Discussion

Fexinidazole has proven to be the ideal drug to be used in treating HAT-infected patients. Its status as a model HAT drug is due to its observed efficacy in treating both the early and late stages of Gambiense as well as Rhodesiense HAT. However, trypanosome resistance to the drug seemed to be generated with relative ease in the laboratory in the Sokolova et al. study(74). FxR parasites for the experiment in this paper will be generated using Sokolova's techniques as well. Although this resistance was deliberately induced in vitro and is not fully predictive of actual human resistance, potential parasitic resistance is still an issue that must be taken into account. The use of eflornithine, a chemically unrelated drug to Fexinidazole, as a partner drug in a combination therapy is a possible solution to this issue. Depending on the results obtained from the experiment, the combination of fexinidazole with eflornithine will, hopefully, either have a synergistic toxic effect on the trypanosome cell or an additive one. Although the additive toxic effect of eflornithine will not preclude the use of the combination therapy, a synergistic effect is preferable as this will contribute even more to the advantage of the combination therapy. Additionally, this experiment will be carried out according to FDA protocols for product development under the animal rules(76). Therefore, two mice from each
group will be sacrificed and necropsied to ensure that the two drugs, when used in conjunction with each other, do not have or cause any unforeseen adverse effects and is well-tolerated.

Fexinidazole has been effective in treating both the early and late stages of sleeping sickness in mice \textit{in vivo} at 100mg/kg or 200mg/kg depending on the subtype of the disease\cite{74}. The use of a 200mg/kg dose in the conduction of the proposed experiments is based on this finding. However, further testing may prove that the combination therapy works just as well with a reduced dose. It is also possible that a higher dose of Fexinidazole will be more effective. Although this is unlikely, the 50\% lethal dose of Fexinidazole of >10,000 mg/kg means that toxicity of this higher dose will be low if present at all.

\textit{Trypanosoma brucei brucei} parasites are going to be used in this study because they are bloodstream forms commonly used in experimental models for HAT. Future studies should be done with \textit{T.b gambiense} and \textit{T.b rhodesiense} trypanosomes to ensure that the combination therapy has the same effect as that observed on the bloodstream \textit{T.b brucei} parasites. These future studies should also observe the effect of the combination therapy on both the early and late stages of HAT.

\textbf{Conclusion}

It is thought that nitroheterocyclic drugs are particularly vulnerable to the development of drug resistance because they rely on only one enzyme for activation\cite{71}. According to the cross-resistance study from which the experimental protocol for generating FxR cells was derived, the ability to generate FxR and NfxR (nifurtimox-resistant) trypanosomes with relative ease supports this hypothesis. Therefore, a combination therapy to reduce potential resistance
to Fexinidazole is imperative as parasitic resistance to the drug will have serious consequences on the use of nitroheterocyclic drugs against HAT in the future along with current treatments for the disease (71). If the proposed results from this experiment are obtained, then this would prove eflornithine’s potential as the ideal partner drug to be used in conjunction with Fexinidazole in a combination therapy because it would either have an additive or synergistic effect against *T. brucei* parasites while also reducing potential resistance to the drug. However, before the combination therapy can be used clinically, its safety must be established according to the preexisting FDA requirements for establishing the safety of new drug and biological products (76).

There is no prophylactic treatment for sleeping sickness, instead, its control is based primarily on chemotherapeutic measures. The discovery of Fexinidazole and its status as a potential ideal drug against HAT once it completes clinical trials was a breakthrough in the control and management of the disease. It is therefore, likely that the drug will experience long-term usage in treating sleeping sickness. However, with long-term monotherapies, the potential for development of parasitic resistance is more likely and can be countered by using partner drugs for a combination therapy. This thesis was designed to show eflornithine’s potential as the partner drug for Fexinidazole. If the partnership is shown to be effective, then the combination therapy will contribute greatly towards the WHO goal of HAT elimination by 2020 (2).
References


